

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/68	A1	(11) International Publication Number: WO 90/04650 (43) International Publication Date: 3 May 1990 (03.05.90)
<p>(21) International Application Number: PCT/GB89/01263</p> <p>(22) International Filing Date: 20 October 1989 (20.10.89)</p> <p>(30) Priority data: 8824592.3 20 October 1988 (20.10.88) GB</p> <p>(71) Applicant (for all designated States except US): ROYAL FREE HOSPITAL SCHOOL OF MEDICINE [GB/GB]; Rowland Hill Street, London NW3 2PF (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : FISHER, Derek [GB/GB]; Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF (GB). FRANCIS, Gillian, Elizabeth [GB/GB]; ANDERSON, Robert [GB/GB]; Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF (GB).</p>		<p>(74) Agents: CRESSWELL, Thomas, Anthony et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5EU (GB).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p> <p>Published With international search report.</p>
<p>(54) Title: A PURIFICATION PROCESS FOR COVALENTLY BOUND DNA/PROTEIN COMPLEXES</p> <p>(57) Abstract</p> <p>A process for separating covalent DNA/protein complexes from non-covalent DNA/protein complexes and unbound DNA comprising the steps of: (i) treating the DNA/protein complexes with a reactive derivative of polyethyleneglycol (PEG) and (ii) subjecting the production of step (i) to phase partition between an aqueous PEG solution phase and an aqueous phosphate solution phase.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

A purification process for covalently bound DNA/protein complexes.

The present invention relates to processes for purifying and estimating covalent DNA/protein complexes and to materials produced by the process.

Current methods to estimate the formation of DNA/topoisomerase and other covalent DNA/protein complexes are time consuming and have other disadvantages. The alkaline elution technique of Zwelling, L.A. et al., Biochemistry, 20:6553-6563 (1981) for detection of protein associated DNA breaks is very lengthy and difficult to standardise. It is also difficult to adapt this technique to recovery of DNA/protein complexes. The precipitation method of Trask, D.K. et al., EMBO J., 3:671-676 (1984) is also a multi-step procedure and although it can be used to recover complexes has the disadvantage that it is known to be most efficient for sections of DNA with many protein molecules in situ and very inefficient for single sites. This probably favours replication fork DNA, since topoisomerase II is very active here. Trask's method does precipitate predominately newly replicated DNA. Site-specific topoisomerase cleavage occurs as either clustered or single sites, and may therefore be under-represented in this method.

Phase systems of immiscible aqueous solutions of polymers and/or salts provide a means of separating cells and macromolecules on the basis of their differential affinities for the two component phases [Walter H, Brooks DE, & Fisher D. (eds.) Partitioning in aqueous two-phase systems, theory, methods, uses and applications in biotechnology. Academic Press, Orlando 1985].

It has now surprisingly been discovered that, by coupling polyethylene glycol (PEG) to the protein component of DNA/protein complexes, covalently bound DNA/protein complexes may be separated from non-covalently bound

- 2 -

DNA/protein complexes and unbound DNA using a suitable aqueous biphasic system comprising a top phase of PEG solution and a bottom, phosphate solution phase.

The present invention therefore provides a process for separating covalent DNA/protein complexes from non-covalent DNA/protein complexes and unbound DNA comprising the steps of:

- (i) treating the DNA/protein complexes with a reactive derivative of PEG and
- (ii) subjecting the product of step (i) to phase partition between an aqueous PEG solution phase and an aqueous phosphate solution phase.

Preferably covalently bound DNA/protein complexes are recovered from the aqueous PEG phase.

Without wishing to be bound by theory, it is believed that the PEG coupling conditions and/or the phase partitioning conditions result in dissociation of non-covalently bound DNA/protein complexes and, in view of the high affinity of DNA for phosphate solutions, [Albertson P-A. Partition of cell particles and macromolecules. 3rd Edition, John Wiley and Son, New York, 1986], it is only the PEG-linked covalent DNA/protein complexes which are drawn to the PEG phase.

Coupling of PEG to the DNA/protein complexes is preferably conducted by reacting the complexes with a reactive 2,2,2-trifluoroethanesulphonyl (tresyl) derivative of PEG, preferably tresyl monomethoxyPEG (TMPEG) which is described in our British Application No. 8824591.5.

The present invention will be further described with reference to the Figures of the accompanying drawings in which:

Fig. 1. shows schematically the coupling of PEG to DNA/protein complexes.

- 3 -

Fig. 2. is a graph showing the effect of sonication on the molecular weight of salmon sperm DNA, (12μ peak to peak amplitude, 20kHz150W).

Fig. 3. is a graph showing the effect of sonication on protein-free DNA partitioning.

Fig. 4. shows graphically the effect of extra rounds of phosphate extraction on recovery of DNA from the PEG phase.

Fig. 5 and Fig. 6 show the dose related increment in PEG phase DNA following treatment of cells with VP-16-213 (Fig 5) and UV (Fig.6).

Fig. 7, 8, 9, 16 and 21 shows scanning densitometer plots.

Fig. 10. is a graph of yields from PEG phase separation of Trask preparations.

Fig. 11. shows differential DNA recovery from retinoic acid induced and uninduced cells.

Figs. 12 and 13 shows results of differential hybridisation experiments.

Figs. 14 and 15 shows results of differential hybridisation experiments.

Figs. 17 and 18 shows the effect of retinoic acid and novobiocin on yield of DNA produced by the Trask method (Fig. 17) and the present method (Fig. 18).

Figs. 19 and 20 shows tubulin hybridisation to fractionated DNA from various induced or uninduced cells.

Figs. 22 and 23 show hybridisation signals from cloned DNA prepared by the process of the invention.

Fig. 24. shows increments in PEG phase DNA from induced or uninduced cells exposed to VP16.

Fig. 25. shows the effect of m-AMSA on DNA yield when partitioned according to the invention.

Fig. 26. shows the effect of VP16 on PEG-phase yield from cells pretreated with retinoic acid.

Fig. 27. shows the use of UV cross-linking to demonstrate retinoic acid induced DNA protein association.

The process of the invention exploits two phenomena (shown schematically in Figure 1). First, activation of PEG with tresyl chloride (panel 1, Figure 1) allows the PEG to be covalently attached to proteins (panel 2). This gives DNA/protein complexes very high affinity to the PEG-rich top phase, allowing the attached DNA to be recovered, while unattached DNA remains in the phosphate-rich bottom phase (panel 5). This can be used to recover or quantitate the protein-linked DNA. The level of DNA in the top (PEG) phase is dependent on the level of DNA/protein complexes in the sample, on the average size of DNA attached to protein (the latter is controlled for some applications by sonication, DNase treatment, or restriction endonuclease digestion of the sample).

Secondly, the reaction mixture and/or phase partitioning conditions described here result in the non-covalently bound protein (with which the majority of eukaryotic DNA is associated) becoming detached from the DNA.

- 5 -

The method therefore results in DNA that was unbound or non-covalently bound to protein being left in the phosphate phase. PEG is known to compact and alter the conformation of DNA. Although the precise nature of this change is still debated, we presume that detachment of non-covalently bound DNA occurs because of changes in the dimensions of the major and minor grooves of DNA (since these are critical for the non-covalent binding of proteins to DNA).

With the new partitioning method the presence of even single topoisomerase molecules will theoretically allow fractionation. The ability of a single PEG-modified topoisomerase molecule, or similar protein, to carry its attached DNA to the PEG phase, depends on the length of attached DNA, since it reflects the outcome of opposing forces (the DNA and PEG-protein compete to take the complex to the phosphate and PEG phases respectively). Successful fractionation of DNA attached to single topoisomerase molecules will therefore only be achieved if the attached DNA is short enough. This can be accomplished by sonication but we have also established the feasibility of using restriction enzyme of DNase digestions while the topoisomerase II is still attached to the DNA. Shorter DNA fragments do, however have lower partitioning coefficients (Muller M Partitioning of nucleic acids. pp 227-266 in: Walter H, Brooks DE, & Fisher D. (eds.) Partitioning in aqueous two-phase systems, theory, methods, uses and applications in biotechnology, Academic Press, New York 1985) i.e., show a more equal distribution between the two phases, so additional rounds of extraction with the phosphate phase (or counter-current phase partitioning) will be required to compensate for this. We have shown that this is easily accomplished.

The present invention may be exploited in, for instance, the following:

- 6 -

- 1) Purification of DNA adjacent to classes of inducible genes (those where induction of down-regulation of transcriptional activity is accompanied by action of DNA topoisomerase I and/or II or other enzymes forming covalent intermediates with DNA). This provides a novel strategy for DNA cloning of inducible genes.
- 2) Purification of DNA at protein binding sites (for proteins not naturally covalently linked to DNA as they act, an additional step is required to attach the DNA to the protein after binding).
- 3) Assay of DNA topoisomerase activity.
- 4) Assay of DNA topoisomerase cleavage-site specificity.
- 5) Preparation of DNA protein binding sites using the "DNase protection" principle.
- 6) Assays for DNA-protein cross linking agents .
- 7) Assays for inhibitors of DNA topoisomerase and other nicking/closing enzyme.

The invention further provides products comprising a DNA fragment covalently bound to a protein having at least one PEG moiety bound to the protein. Preferably the PEG moiety is a monomethoxy PEG group.

- 7 -

THE INVENTION WILL NOW BE ILLUSTRATED IN THE
FOLLOWING EXAMPLES.

EXAMPLES 1 TO 11

a) ACTIVATION OF PEG WITH TRESYL CHLORIDE

To avoid hydrolysis of tresyl chloride all reagents must be dried before use. Monomethoxy poly(ethylene)glycol (MPEG) (Mr 5000, Union Carbide, USA) was dissolved in benzene (B.P. 79-80°C) and the water-organic azeotrope (B.P. 65°C) was distilled off. MPEG was recovered by removal of solvent under reduced pressure and was finally dried by leaving overnight at room temperature under vacuum. Dichloromethane (AnalaR grade BDH, U.K.) was dried over molecular sieve A3 (crystalline potassium alumina silicate, BDH, U.K.) overnight at room temperature using 100g per litre of solvent.

Dry MPEG (18g, 3.5mmol) was dissolved in dry dichloromethane (45ml) at room temperature. The mixture was cooled to 0°C, stirred magnetically and 1.125ml (14mmol) of pyridine (AnalaR grade BDH, U.K.) and 1ml (9mmol) of tresyl chloride (Fluka AG, Switzerland) at 0°C were added dropwise. The reaction was allowed to continue at room temperature with constant stirring for 1.5h before the dichloromethane was removed by evaporating under reduced pressure. The white solid was dried under vacuum overnight at room temperature.

This tresyl-PEG preparation (TMPEG) was washed to remove pyridine before use. The TMPEG was dissolved in methanol-HCl mixture (250:1) and allowed to precipitate -20°C for 8 h. The resulting white solid was collected at 0°C and the filtrate checked for pyridine content spectrophotometrically (λ_{max} 255 nm). This procedure was repeated by using methanol-HCl (1000:1) as washing mixture until no pyridine could be detected. Finally, the pyridine-free TMPEG (10-12g; 55-66% yield) was dried under vacuum for several hours at room temperature. Sulphur content was 0.5%

- 8 -

(theoretical content for 1 tresyl group per molecule of MPEG-5000 is 0.62%). The TMPEG was stored desiccated at 4°C prior to use.

b) PEG MODIFICATION OF DNA/PROTEIN COMPLEXES

TMPEG, 400mg/ml in 0.05M Na phosphate 0.125 NaCl buffer pH7.5 was mixed with cell lysate or DNA/protein complexes at a ratio 1:1 (v:v) on a rotating mixer for 2h at room temperature. Since we do not know the number of lysine molecules per molecule of topoisomerase II and there are other proteins present, we chose to add TMPEG in excess. Experiments with albumin indicate maximum partitioning to the PEG phase with a TMPEG:lysine molar ratio 8:1 and significantly increased partitioning was achieved at much lower values. The ratios of DNA/protein complexes to TMPEG used in these experiments therefore represent a gross excess of TMPEG, but this may have additional advantageous effects because of the effects of PEG concentration on DNA conformation (4,5). Further experiments will be needed to evaluate these two facets of the separation procedure independently (using mixtures of TMPEG and PEG during the covalent modification step).

Aliquots of TMPEG treated material were added directly to the phase system. Where further enzymatic treatments (e.g. with restriction enzymes) are planned it may be advisable to neutralise unreacted TMPEG to prevent it modifying these proteins. We have established, in our work on immunoaffinity phase partitioning, that lysine or albumin can be used to prevent further undesired reaction of the TMPEG after the coupling step. In practice we found that proteinase K is robust and, provided overnight incubations at 37°C are used, is little affected by the addition of TMPEG-treated material. However, for all other procedures using a second enzymatic procedure, 1M free base lysine (Sigma)

- 9 -

dissolved in coupling buffer (1 vol) was added to the reaction mixture (6 vols) and the mixture incubated at room temperature for a further hour.

c) PHASE PARTITIONING

The phase system chosen consists of 10% (w/w) poly(ethylene glycol) 6000 (BDH). 14% phosphate (ratio of 16.86g KH_2PO_4 and 40.20g $\text{K}_2\text{HPO}_4(3\text{H}_2\text{O})$ and 76% distilled deionised sterile water. The system was allowed to mix and settle into an upper PEG-rich and a lower phosphate-rich phase. These were separated and individually filtered through 0.22um filter (Gelman Science Inc. Michigan) aliquotted and stored at -20°C . This was done because it is difficult to sample aliquots of the mixed phase system without taking varied proportions of the two phases and this would reduce the reproducibility of the method. For each fractionation, the phase system was reconstructed usually using the stated volume ratio of PEG:phosphate phases, with no more than 15% of the total volume of the phase system consisting of tresyl-PEG treated DNA/protein extract. For experiments fractionating small DNA fragments the ratio of PO_4 :PEG phases was increased usually to 750:250ul and multiple rounds of phosphate extraction were used. The phases were mixed by vortexing and allowed to settle at 25°C for 10 min. For multiple phosphate extractions (see above) the PEG rich phase was then transferred to a fresh phosphate phase and the procedure repeated (the number of rounds of extraction is indicated with each experiment).

d) SONICATION OF DNA OR DNA/PROTEIN COMPLEXES

TO REDUCE THE AVRAGE LENGTH OF DNA

Pure salmon sperm DNA (used for the experiments in Example 1) was diluted to 10 ug/ml in TE (10mM TrisCl, 1mM EDTA, pH7.4) and 500ul aliquots were sonciated at 12u peak to peak, 20 KHz, 150 watts in an MSE bench sonicator on ice, taking care

to avoid frothing. For all other experiments, 500ul aliquots of tresyl-PEG treated HL60 cell DNA/protein lysate (containing protease inhibitors - see above) were sonicated for 5-60 seconds, using the same settings as above.

e) DNase TREATMENT OF DNA/PROTEIN COMPLEXES
TO PARTITION "PROTECTED DNA"

For the experiments of Example 6 DNA/protein complexes equivalent to 2.5ug DNA (prepared by the Trask method) were exposed to 10ug DNase I (bovine pancreatic DNase, Sigma Ltd.) in 500ul of reaction mixture. The latter contained 50M Tris HCl; 25mM MgCl₂; 20mM Cl; 1mM CaCl₂; 10% w/v glycerol; 50ug/ml bovine serum albumin (BSA - Sigma Ltd.). After the exposure times indicated, the reaction was stopped using a final concentration of 1% SDS and 15mM EDTA.

Where DNase was used as an extra step between two rounds of partitioning, recovered DNA/protein complexes in 160ul PEG-phase were exposed to 2.5ug of bovine pancreatic DNase I in the presence of 65ul Tris EDTA pH 8.0, 12.5ul of 50mM MgCl and 12.5ul of 50mM CaCl₂ and 10ul of 10% BSA. Two minutes exposure was used

- 11 -

f) RECOVERY OF DNA FROM THE PHASES AND QUANTITATION

We evaluated several means of estimating the amount of DNA in the phases. Many commonly used methods are not applicable to the DNA while in the phases themselves. Chemiluminescence prevented use of [H^3]DNA and scintillation counting. The fluorescence of PEG prevented the use of Hoechst 33258 (an intercalating dye used to estimate DNA by fluorescence). PEG can be removed by chloroform extraction and phosphate and other salts by an appropriate desalting column.

The level of DNA in the PEG phase serves as a measure of the DNA complexed to topoisomerase, but this cannot readily be measured fluorimetrically in the presence of the protein because of quenching. Phenol/chloroform extraction is used to separate protein from DNA, but cannot be used directly because protein covalently linked to DNA will cause loss of the attached DNA (usually the DNA desired). The topoisomerase must therefore be digested. Proteinase K (Sigma Chemicals Poole) 20mg/ml aqueous solution was added (100ul/ml of PEG or phosphate phase) and incubated at 37°C overnight. This step can then be followed by an extraction procedure to recover the DNA (e.g. by three rounds of phenol/chloroform extraction using a 1:1 ratio (v/v) (phenol/chloroform contained 0.1% 8-hydroxyquinoline - BDH Chemicals Poole) and one round of chloroform/isoamyl alcohol (24:1 V:V) to remove any remaining phenol. This procedure effectively removes PEG but not phosphate. In order to remove the latter (since ethanol precipitation of DNA is not effective if salt concentrations are too high) 110ul of the extracts from both PEG-rich and phosphate-rich phases were loaded onto 1.2ml columns of Sephadex G50 (Pharmacia) packed in a 1ml syringe. The Sephadex was pre-equilibrated with 10mM Tris HCl, 100mM NaCl 1mM EDTA pH 8.0 (STE). Columns were spun at 400g for 4 min. DNA was recovered from the

- 12 -

eluate by precipitation in 2 volumes of absolute alcohol at -70°C for 1 hour followed by centrifugation at 11,000g for 10 min. in a microfuge (MSE instruments) and the pelleted DNA resuspended in 20ul of Tris EDTA: 10mM Tris:HCl, 1mM EDTA pH7.4 (TE). This DNA can then be estimated by conventional means. For the examples above it was either estimated by ultraviolet absorbance spectrophotometry, or, where we wished to examine the size distribution of the loaded and recovered DNA (since partitioning behaviour varies with DNA fragment size), we used agarose gel electrophoresis. A 6ul aliquot of each sample was run in a 1% (w/w) agarose gel (100x75mm) with ethidium bromide 1ug/ml in Tris running buffer: Tris borate 0.089M, boric acid 0.089M, 0.002M EDTA (TBE) at 24 volts (2.4 V/cm) for 1 hour. The gel was photographed on a UV transilluminator at 245nm with Polaroid 667 film. The DNA was estimated from the integrals of scanning densitometer plots of the negatives (Joyce Lobel Instruments Gateshead) by comparison with known amounts of DNA. Experiments adding known amounts of DNA individually to PEG and phosphate phases with this procedure showed that recoveries vary for the two phases. For a rapid quantitation samples were spotted onto an agarose slab, photographed on a transilluminator as above and compared to a set of DNA standards.

Losses are significantly lower for the PEG than for the PO_4 phases (see Example 1). Thus to estimate the "true" partitioning behaviour of samples, results have to be corrected for this differential loss and recovery standards were included for this purpose in specified experiments.

When calculating recoveries with respect to loaded DNA to estimate the DNA lost in the procedure, in order to exclude losses for subsequent steps (PK digestion phenol/chloroform extraction ethanol precipitation etc.) an aliquot identical to the DNA loaded was treated as the recovered samples and used as a reference against which to

- 13 -

calculate the % loaded DNA recovered.

Preparation of HL60 cell protein-associated DNA

HL60 cells in log phase growth were harvested by centrifugation at 400g for 6 min. and resuspended in serum free medium (RPMI Gibco Ltd.) at 5×10^6 cells/ml. Cells were lysed in the presence of protease inhibitors by the addition of 10% aqueous SDS to a final concentration of 1% with 1% v/v Triton X-100 (Sigma), 15mM EDTA and 1mM phenylmethylsulphonylfluoride (Sigma) from a stock solution of 100mM in methanol. The cell lysate was vortexed aliquotted and stored at -20°C .

In some experiments cells were pre-treated for 70 min with 10^{-6}M all trans retinoic acid (sigma) or VP16-213 10^{-5}M (Vepesid, Bristol Meyers U.K.) for the times stated. The timing of retinoic acid treatment was selected because previous studies indicate that protein associated DNA breaks induced by differentiation are present after this time interval. Controls were sham treated with the relevant diluent for the same times. Appropriate sham treatment of controls is particularly important in some types of experiment (e.g. UV protein crosslinking) where cell manipulations clearly influence DNA/protein associations and hence yields. For such experiments, individual results must be expressed with respect to matched sham treated controls rather than, say, to a single "untreated" or time zero controls.

SDS/KCl precipitation of DNA/protein complexes (Trask procedure)

This method is essentially that given in the literature (loc. cit.) except that calf thymus DNA was omitted from the binding reaction buffer and vigorous vortexing was not used (so that long DNA fragments were not sheared).

g) INCREASING DNA-TOPOISOMERASE CROSSLINKING WITH VP16

- 14 -

Log phase HL60 cells, grown in RPMI 1640 supplemented with 10% foetal calf serum (both Gibco - Paisley Scotland), were harvested and resuspended to 6.5×10^6 /ml. The cells were then allowed to re-equilibrate in the incubator at 37°C.

Etoposide (VP 16-213 Bristol Meyers, NY state USA) was serially diluted in serum free RPMI with an appropriate diluent control, to final concentrations of 10^{-5} M down to 10^{-9} M and an equal concentration of diluent through the gradient. This was then allowed to react with the cells for 15 mins at 37°C. The reaction was finally stopped by the addition of sodium dodecyl sulphate and Triton X-100 (both Sigma Chemicals - Poole England) to a final concentration of 1% (v/v). Phenyl methyl sulphonyl fluoride (PMSF) and disodium ethylenediaminetetraacetic acid (EDTA both Sigma Chemicals - Poole England) were also added at a final concentration of 1mM and 15mM respectively.

Tresyl monomethoxy poly(ethylene)glycol (TMPEG) was dissolved in coupling buffer (0.5M NaPO₄ 0.08M NaCl pH 7.5) to a concentration of 400mg/ml and an equal ratio (200ul: 200ul) was allowed to react with each of the cell lysates on a rotating turntable at room temperature for two hours.

Six 1000ul phases were constructed (500:500ul) from PEG and Phosphate and 100ul of the TMPEG:cell lysate mixture was added to each phase. These were then vortexed and allowed to settle at 25°C for 10 mins. The phases were then separated and incubated at 37°C overnight with proteinase K (Sigma Chemicals - Poole England) at a final concentration of 100ug/ml. The phases were then cleared of protein by extraction using two rounds of phenol/chloroform with 0.5% 8-Hydroxyquinoline (all BDH Chemicals - Poole England).

An equal aliquot (10ul) of every sample was run for one hour

- 15 -

at 25v/cm through a 1% agarose gel with Tris borate running buffer (0.089M Tris borate, 0.089M boric acid, 0.002M EDTA (pH 8.0) all BDH chemicals - Poole England). The gel was then photographed under ultra violet light of wavelength 254nm using Polaroid 667 instant film. The negatives were scanned with a Joyce Lobel densitometer and the resultant integral's were used to calculate the DNA recovered with respect to the untreated control.

h) UV CROSSLINKING OF NON-COVALENTLY BOUND PROTEINS TO DNA

Reference is Gilmour and Lis PNAS 81 p4275-4279;1984

Log phase HL60 cells growing in RPMI1640 medium supplemented with 10% foetal calf serum (both Gibco) were taken and spun at 400g for 4 minutes. These cells were then resuspended to the required concentration. A 1ml aliquot was then placed in a small petri dish (Nunc products) and exposed to UV light at a wavelength of 254 nm and intensity 5W/cm² at 12.5cm. Cells were then collected by centrifugation in an Eppendorf tube at 6500g for 5 minutes. The cells were resuspended in 200 microlitres 10 mM Tris HCl pH 7.4 1mM EDTA (T.E.) sodium dodecyl sulphate, SDS (Sigma) and Triton X - 100 (BDH) to a final concentration of 1% and disodium EDTA (Sigma) was added to a final concentration of 15 mM. The samples were then cooled to 4°C and sonicated with an MSE bench sonicator on ice for 30 seconds, 12 micrometers amplitude peak to peak, 150 Watts and 20 kHz. This reduces the modal size of the DNA to about 800 basepairs. The negative control sample was incubated at 56°C for 1.5 hours with proteinase K (Sigma) to a final concentration of 5 micrograms per microlitre. This sample was then treated as the others.

Tresyl methoxypoly(ethylene glycol) TMPEG was prepared to 400 mailgrams per millilitre buffer in 0.05 sodium phosphate 0.125 sodium chloride pH 7.5 and was mixed

SUBSTITUTE SHEET

- 16 -

in a ratio of 1:1 (v/v) with the U/V irradiated and unirradiated samples for 2 hours at room temperature.

After incubation with TMPEG all the material was added to a phase system composed of 750 microlitres phosphate-rich and 250 microlitres PEG-rich phases. The system was constructed using 14.7 % w/w poly (ethylene glycol) 6000 (BDH), 11.2% w/w phosphate (ratio of 16.86 grams KH_2PO_4 and 40.20g K_2HPO_4 ($3\text{H}_2\text{O}$) both BDH) and 74.1% distilled deionised sterile water. The phases were vortexed and allowed to settle for 10 minutes at 25°C after which the upper PEG-rich phases were removed to fresh 750 microlitre phosphate rich bottom phases. This was then repeated to leave one top phase and four bottom phases per condition.

Each condition was then incubated at 37°C O/N with proteinase K to a final concentration of 5 micrograms per microlitre. The phases were then extracted with two equal volume rounds of phenol-chloroform (phenol:chloroform 1:1 v/v containing 0.1% hydroxyquinoline, all BDH) and a final extraction by chloroform-isoamyl alcohol 24:1 (both BDH) to remove any residual phenol. This removes any PEG present but not any phosphate. The phosphate must be removed as Ethanol precipitation of DNA is ineffective in high salt concentrations. The phosphate is removed spinning a sample down a 1 ml syringe packed with Sephadex G-50 (Pharmacia) which has been equilibrated with STE pH8 (10mM Tris HCl pH8; 1mM EDTA; 100mM NaCl). The eluate is then precipitated with two volumes of cold absolute alcohol at -70°C overnight.

The DNA was then spun for 10 minutes at 11,000 r.p.m. in a MSE microcentrifuge and resuspended in 10 microlitres T.E. pH 7.4. This was then run on a 1% agarose gel for 2 hours at 2.5 volts per centimetre. The gel was photographed on a UV trans-illuminator with Polaroid 665 film. The negatives scanned with a densitometer (Joyce Lobel) and the integrals were used to calculate the amount of DNA present

- 17 -

using a known salmon sperm standard.

The results were plotted as DNA present in the top phase as a percent of the starting amount and also as the amount in micrograms recovered from the top phases.

i) PRE-EXPOSURE OF CELLS TO NOVOBIOCIN TO INHIBIT DNA TOPOISOMERASE II AND HENCE REDUCE DNA ATTACHMENT

HL60 cells were pre-incubated with 10^{-3} M novobiocin (Sigma Ltd.) for 60 min at 37°C under standard culture conditions (see above) and were then either exposed to retinoic acid (10^{-6} M for 70 min) or sham treated with diluent. Toxicity tests showed that less than 5% cells died with this dose of novobiocin when assessed by nigrosin. Novobiocin is an established inhibitor of DNA topoisomerase II that acts in a different manner to the epipodophyllotoxins and intercalators, in that, unlike the latter it does not stabilise cleavable complexes of enzyme and DNA, but tends to inhibit binding of the enzyme to DNA. However it was recently shown that the agent has a complex interaction with the enzyme in that with alterations in the ratio of drug:enzyme both inhibitory and stimulatory effects can be observed (Collins and Johnson Nucl Acids Res 7:1331;1979)

j) DIFFERENTIAL HYBRIDISATION

The hybridisation strategy using PEG and phosphate phase DNA from matched differentiation induced and uninduced cells is explained in Example 7.

The filters were prepared by the dot blot method of FC Kafatos, CW Jones and A. Efstratiadis. Nucleic Acid Research 7:1541;1979. Lifted from The Guide to Molecular Cloning Techniques, edited by S.L. Berger and A.R. Kimmel Academic Press 1987.

- 18 -

1) Preparation of nitocellulose or nylon GeneScreen Plus filters (New England Nuclear Research Products - Boston USA) with DNA from DNA/protein complexes recovered from cells induced by all trans retinoic acid or phorbol-12-myristate 13-acetate (PMA) (both Sigma Chemicals - Poole England).

Usually 4 dilution of DNA were added to the filter with 0.05 ug as the highest dilution and three subsequent dots with doubling dilution. Two to three replicate filters were made with independent loading denaturing etc. Samples were diluted with T.E. pH 8.0 (10mM Tris.Cl 1mM Ethylenediaminetetraacetic acid, EDTA) to 0.1ug of DNA. Each sample then had addition of TE pH 8 to bring the volume to a standard 50ul. All the samples were heated to 70°C with 0.1 volume of 3M sodium hydroxide (BDH Chemicals - Poole England) for 45 minutes. This denatured the DNA and destroyed any RNA, if present. The samples were then allowed to cool to room temperature and 55ul 2M ammonium acetate (BDH Chemicals - Poole England) was added. Serial dilutions were then carried in 1M ammonium acetate. The DNA was loaded onto the nylon (or nitro cellulose) with a 'home made' Dot - blot device and allowed to stand at room temperature for at least 30 minutes prior to being sucked through by vacuum. Filters were then dried between 3MM paper (Whatman Paper - Maidstone England) and then baked for two hours at 80°C under a vacuum (optional for nylon filters). The filters were then pre-hybridised, using 6x SSC, (0.9M Saline, 0.9M Sodium Citrate pH 7.0 Both BDH Chemicals - Poole England), 0.5% SDS, (Sodium Dodecyl Sulphate, Sigma Chemicals - Poole England) 5x Denharts solution (0.5g Ficoll, 0.5g polyvinylpyrrolidone and 0.5g Bovine Serum Albumen Pentax Fraction V - all Sigma Chemicals, Poole England) and 100mcg/ml of highly sonicated salmon sperm DNA (Sigma Chemicals - Poole England) in 200mls of distilled deionized water. The filters were prehybridised

- 19 -

for 3.5 hours at 68°C, with agitation, in the shaking waterbath. After pre-hybridisation, the fluid was poured off and hybridisation fluid was added which consisted of 6x SSC, 0.5% SDS, Denharts, 100 mcg/ml denatured salmon sperm DNA and 0.1M EDTA in 100ml volume. To this was added a probe radio labelled with dCT³²p, using the random primer method kit (Amersham Plc - Amersham England) and the amount of incorporation was assessed using adsorption to Whatman DE-81 filter paper Manniatis loc.cit. with washing to remove free nucleotides. Hybridisation was allowed to proceed again at 68°C with agitation for about 17 hours. Post hybridisation washes were twice 45 minutes and then twice 30 minutes of 1x SSC, 1x Denharts and 0.1% SDS in 100ml distilled deionized water which had been pre warmed to 68°C. This solution was added to the filters after the hybridisation fluid had been removed. The post hybridisation washes were also carried out with agitation in the waterbath. These washes were then followed by two final 20 minute rinse washes also with agitation in the waterbath with 100ml of pre warmed 0.1x SSC at 68°C. The filters were then blotted between sheets of Whatman 3MM paper and then allowed to dry thoroughly in air. The filters had a count of no more than 2-5 cpm above background and were exposed to Fuji RX 100 medical X - ray film (Fuji Photo Film Co. Ltd. - Japan) with two rare earth intensifying screens (Du Pont Cronex Quanta III, Du Pont Industries U.K.) at -70°C for 24-48 hours depending upon the result obtained.

The probes used included c-myc a 8Kbp ECo R1/Hind III fragment of genomic DNA (Dalla-Faura et al PNAS. 79:6497;1982.) as well as a 1.5Kbp cDNA fragment of c-fos (truncated with respect to the 5' end, lacking the 1st exon) (Murphy and Norton personal communication) as well as a retinoic acid plus (R+) probe which was prepared as follows. Retin ic acid induced cells were used to produc protein

- 20 -

associated DNA, prepared by the method of Trask et al. This material (5.0ug) was then exposed to 0.05 ug Bovine Pancreatic DNase 1 (Sigma Chemicals - Poole England) for a period of five minutes in the presence of 50mM Tris.Cl pH 8.0, 10 % v/v Glycerol, 0.1% Bovine Serum Albumen, 250mM Mg^{2+} 10mM Ca^{2+} and 200mM K^{+} (all Sigma Chemicals Poole - England) in 250ul volume. A similar probe (R+) was prepared from analogous, undifferentiated HL60 cells.

The reaction was stopped by bringing the reaction mixture up to 1% SDS and 15mM EDTA. An equal volume of 400mg/ml TMPEG (tresyl monomethoxy polyethylene glycol) was allowed to react to the protein present for two hours at room temperature and then was partitioned on a 250/270 ul PEG:PO₄ phase system with five rounds of PO₄. The DNA was then liberated from the protein by the addition of proteinase K (Sigma Chemicals - Poole England) to 20mg/ml overnight at 37°C. The samples were then extracted with two rounds of phenol/chloroform followed by a single extraction with isoamyl alcohol/chloroform.

k) Cloning of topoisomerase-associated DNA prepared by the new procedure

Two different cloning techniques were evaluated, but in principal any standard technique could be used.

a) The homopolymer tailing technique was used to introduce the prepared DNA fragments into dCTP tailed plasmid (puc8). This method was selected because no differential handling of topoisomerase cleavage sites, sheared ends or restriction sites is involved (we wanted to avoid differences in efficiency of processing fragments with and without the topoisomerase staggered cleavage sites). Transformation followed by growth of plasmid in and recovery from DH5 E. coli, demonstrated that recombinant plasmids were produced. However the efficiency of this method was relatively low.

- 21 -

b) The second strategy for the cloning of topoisomerase-associated DNA was as follows: the overhang created by topoisomerase II cleavage is endfilled with Klenow (Amersham U.K.) and then Sa I linkers (Pharmacia) ligated to the blunt ends. The DNA is then digested with Eco RI and Sal I (Eco RI to digest the largest DNA fragments down to a reasonable size and Sal I to create sticky ends). The DNA is then ligated to pUC 18 which has been digested with Eco RI and Sal I and transformed into E.coli DH5 cells (BRL). This procedure should clone both randomly cleaved ends of DNA as well as the topoisomerase associated DNA, but will tend to exclude Sal I/Sal I fragments cut from sections of DNA distant from the topoisomerase II cleavage site.

Using Xgal plates, for some unknown reason this material gives many blue colonies which are produced on transformation in addition white colonies. This was despite having ligated vector alone and seeing that it was very inefficient at producing clones (as expected). The efficiency of producing white colonies varied between batches of ligated vector/inserts. It is possible these blue colonies contain very small inserts and a sample should therefore be examined before these are discarded.

The DNA fractionated by the new method initially proved resistant to digestion with restriction enzymes however this was over come by first passing the DNA through QIAGEN (Trademark) tips. The DNA for cloning by this procedure should be kept as large as possible to reduce the number of randomly cleaved ends available for cloning.

Cloning may be improved by using vector cut with Sal I alone and then treated with calf intestinal phosphatase to remove terminal phosphate groups. If there is a Sal I site between

- 22 -

the artificially created Sal I site at the topoisomerase cleavage site by the attachment of a Sal I linker and the nearest Eco RI site, then these will be cloned (as will as other Sal I-Sal I fragments).

Cloning of DNA (cf. Maniatis *et al*, Molecular Cloning : A Laboratory Manual, Cold Spring Harbor, 1982

1) preparation of DNA for cloning

QIAGEN Tip Purification of fractionated DNA

Dilute sample with water to 130 ul

Add 70 ul 1.5 M NaCl/250mM MPS pH7.0

Equilibrate QIAGEN tip 5 with 300 ul buffer A

Absorb DNA on to tip by pipetting through tip 4 times

Wash off linkers with 5x750 ul buffer C

Elute DNA in 3x200 ul buffer F

Add 0.48 ml (0.8 vol) isopropanol and place on ice 15 min

Microfuge 30 min

Wash pellet with 70% ethanol

Microfuge 30 min

Dry pellet under vacuum

Dissolve in TE pH 7.5

Blunt Ending of DNA

2 ul 10xrepair buffer

5 ul 1mM dNTPs (1 mM each)

6 ul TE pH 7.5

5 ul DNA (2.0 ug)

1 ul Klenow (5U/ul)

Incubate 1 hour at room temperature

Heat at 65°C for 5 min (to heat inactivate enzyme)

Phosphorylation of Linkers

1ul 10xkinase buffer

1 ul linkers (1 ug)

SUBSTITUTE SHEET

- 23 -

1 ul T4 polynucleotide kinase
7 ul ddw (double distilled water)
Incubate 1 hour at 37°C

Ligation of DNA to Linkers

20 ul blunt ended DNA
6 ul 5xligase buffer (BRL)
5 ul kinased linkers
10 ul ddw
5 ul T4 DNA ligase (1 U/ul)

Incubate at 4-15°C overnight
Add 50 ul phenol/chloroform and mix
Microfuge briefly and reextract with phenol/chloroform
Extract with chloroform
Back extract with 50 ul TE pH 7.5
Add 4 ul 5 M NaCl to 100 ul DNA
400 ul 100% cold ethanol
Chill at -70°C then microfuge 15 min
Vacuum dry pellet

Restriction Endonuclease Digestion

40 ul ddw (to dissolve DNA)
5 ul 10xbuffer (BRL H buffer for Eco RI and Sal I)
1 ul Eco RI (90 U/ul)

Incubate at 37°C for 1 hour
Heat inactivate at 68°C for 5 min
Add 4 ul Sal I (10 U/ul)
Incubate at 37°C for 4 hours

QIAGEN Tip Removal of Linkers

Add to 50 ul of DNA
100 ul ddw

- 24 -

25 ul 5 M NaCl

7.5 ul 1 M MOPS pH 7.0

Equilibrate QIAGEN tip 5 with 300 ul buffer A

Adsorb DNA on to tip by pipetting through tip 4 times

Wash off linkers with 5x750 ul buffer C

Elute DNA in 3x200 ul buffer F

Add 0.48 ml (0.8 vol) isopropanol and place on ice 15 min

Microfuge 30 min

Wash pellet with 70% ethanol

Microfuge 30 min

Dry pellet under vacuum

Dissolve in 40 ul of ddw

Run 2 ul in a 0.8% agarose gel to determine average size

2) Preparation of vector for cloning

Preparation of pUC 18

16 ul pUC 18 (0.25 ug/ul ie 4 ug)

2 ul 10xbuffer (BRL H buffer for Eco RI and Sal I)

1 ul Eco RI (90 U/ul)

Incubate at 37°C for several hours

Run 1 ul on a 1% minigel to ensure cut to completion

Add 1 ul Sal I (10 U/ul)

Digest at 37°C for several hours

Add 110 ul ddw, 70 ul 1.5 M NaCl, 120 mM MOPS pH 7.0

Remove small inserts using QIAGEN tips as above

Dissolve in ddw to a concentration of 0.1 ug/ul

Dephosphorylation of Vector

5 ul 10xCIP buffer

45 ul vector (2 ug)

1 ul calf intestinal phosphatase

Incubate at 37°C for 30 min

- 25 -

Add 1 ul CIP and incubate a further 30 min

Add 40 ul ddw

10 ul STE (saline Tris EDTA)

5 ul 10%SDS

Heat at 65°C for 15 min

Add 4 ul 5 M NaCl, 200ul cold ethanol and precipitate DNA at -20°C

Microfuge for 15 min, remove supernatant and dry under vacuum

Dissolve in ddw to a concentration of 0.1 ug/ml

3) Ligation of vector to insert DNA

0.1 ug pUC 18 (cut with Eco RI/Sal I)

Insert DNA (at ratio 5:1 (mol:mol) and others)

ddw to 20 ul

Heat at 68°C for 5 min

Cool on ice

Add 4 ul 5xligation buffer

1 ul T4 DNA ligase

Incubate at 15°C overnight

4) Transformation of E. coli (DH5 competent cells)

As per BRL protocol

Thaw cells on ice

Dispense into 20 ul aliquots

Add 1 ul of vector/insert DNA (diluted 1:5 in TE)

Incubate on ice for 30 min

Incubate at 42°C for 40 sec then place on ice

Add 80 ul SOC and incubate at 37°C for 60 min with shaking (225 rpm)

plate onto X-gal plates and incubate overnight

Pick colonies into 100 ul L Broth + ampicillin and grow overnight

Add 100 ul 30% glycerol and store at -70°C

SUBSTITUTE SHEET

- 26 -

5) Solutions and buffers

TE

1 mM EDTA

10 mM Tris

10xRepair buffer

0.5 M Tris pH 7.4

70 mM MgCl₂

10 mM dithiothreitol

10xKinase buffer

0.66 M Tris pH 7.6

10 mM ATP

10 mM spermidine

10 mM MgCl₂

150 mM dithiothreitol

2 mg/ml BSA (molecular biology grade)

10xCIP buffer

0.5 M Tris pH 9.0

10 mM MgCl₂1 mM ZnCl₂

10 mM spermidine

6) Church hybridizationReference

Church, G.M., and Gilbert, W. (1984) Genomic Sequencing,
Proc. Natl. Acad. Sci. USA 81, 1991-1995

Solutions1.0 M Sodium Phosphate Buffer 7.2

- 27 -

257.0 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
 56.8 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
 Make to 1000 ml with ddw
 (May require heating to dissolve)

or

134 g $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$
 4 ml phosphoric acid
 Make to 1000 ml with ddw
 (May require heating to dissolve)

Church Prehybridization Buffer

0.5 M NaPi pH 7.2

7% SDS

1 mM EDTA

<u>Stock</u>	<u>200 ml</u>	<u>500 ml</u>	<u>1000 ml</u>
1 M NaPi pH 7.2	100 ml	250 ml	500 ml
20% SDS	70 ml	175 ml	350 ml
0.5 M EDTA	0.4 ml	1.0 ml	2.0 ml
ddw	29.6 ml	74 ml	148 ml

Church Washing Solution

40 mM NaPi Ph 7.2

1% SDS

<u>Stock</u>	<u>1000 ml</u>	<u>2000 ml</u>	<u>5000 ml</u>
1 M NaPi pH 7.2	40 ml	80 ml	200 ml
20% SDS	50 ml	100 ml	250 ml
ddw	910 ml	1820 ml	4450 ml

- 28 -

Procedure

Prehybridize filter in 50-100 l/cm² at 65 C in plastic bag 2-6 hours

Add probe and hybridize overnight (16-24 h) at 65 C

Wash 2x5 min in Church wash at room temperature

Wash 2x30 min in Church wash at 65 C

Autoradiograph overnight with intensifying screen

7) Media, plates ad reagentsSOC

2g bactotryptone

0.5 g yeast extract

1 ml 1 M NaCl

0.25 ml 1 M KCl

Autoclave in 97 ml ddw

Cool to room temperature

Add 1 ml 2 M Mg⁺⁺ and 1 ml 2 M glucose

Make to 100 ml

Filter sterilize

L Broth

10 g Bactotryptone

5 g Bactoyeast Extract

10 g NaCl

Made to 1000 ml with ddw

Autoclave to sterilize

Cool then add 1 ml 25 mg/ml ampicillin

L Agar

1.5 g bacto agar in 100 ml L Broth

Auto clave to sterilize

Cool then add 1 ml 25 mg/ml ampicillin and pour plates

- 29 -

X-gal

20 mg/ml in dimethylformamide

100 mg x-gal in 5 ml dimethylformamide

X-gal: 5-bromo-4-chloro-3-indolyl- -D-galactopyranoside

Store at 4 C

0.1 M IPTG

119 mg IPTG in 5 ml sterile ddw

Filter sterilize, then aliquot into microfuge tubes

Store at -20 C

IPTG: isopropyl-thiogalactopyranoside

25 mg/ml Ampicillin

250 mg ampicillin dissolved in 10 ml ddw

Filter sterilize, then aliquot into microfuge tubes

Store at -20 C

X-gal Plates

Cool 100 ml L agar to < 50

Add 400 ul ampicillin at 25 mg/ml

300 ul X-gal at 20 mg/ml

100 ul at 0.1 M

Pour plates. Store at 4 C wrapped in cling film

- 30 -

EXAMPLE 1THE PEG-PROTEIN COUPLING PROCEDURE INCREASES THE AFFINITY OF PROTEIN-BOUND DNA FOR THE PEG PHASE AND ALLOWS RECOVERY AND QUANTITATION OF THAT DNA

Before attempting to demonstrate the extent to which PEG-phase recovery is due to the coupling of PEG to the protein attached to the DNA, it is important to appreciate that PEG-phase recoveries of free DNA are highly influenced by DNA fragment length. This therefore needs to be considered when making comparisons to demonstrate affinity partitioning and also when selecting partitioning conditions (ratio of PEG:phosphate phase volumes and required rounds of phosphate extraction) to render the system more specific for PEG-protein-DNA complexes.

Figs 2&3 shows the influence of sonication on PEG-phase yield. As the molecular weight of DNA falls the yield in the PEG phase increases.

Figure 3 shows the influence of sonication on Peg phase yield using 1:1 PEG:phosphate phase volume ratio and a single round of partitioning (circles) or seven rounds with fresh phosphate phases (squares).

These results are in agreement with those expected on theoretical grounds since with small molecules the partition coefficients approach unity (i.e. equal distribution between the two phases) Muller loc. cit. and the specificity of the partitioning is lost. Given the known partitioning coefficients of PEG-proteins Walter loc. cit. which approach infinity (i.e. 100% in the PEG phase), we anticipated that the desired affinity-partitioning could still be achieved, even if DNA was highly fragmented (and this is often desirable on theoretical grounds - see below), merely by repeatedly extracting the PEG-phase with additional rounds of phosphate-phase (figure 2 squares). To limit the number of rounds required, phase systems can also be constructed with

- 31 -

larger PO_4 than PEG phases.

To establish that additional rounds of phosphate extraction successfully remove small fragments of protein free DNA and leave essentially only protein associated DNA, whole genomic or DNA-protein complex enriched DNA was partitioned with additional rounds of phosphate extraction (a typical example is shown in Figure 3, in which, after 3 rounds of PO_4 extractions very little (0.2%) further DNA was recovered in the PO_4 phase).

Figure 4 shows the effect of additional rounds of extraction with phosphate phase on the recovery of DNA in the PEG and phosphate phases. Results are means \pm SEM of 4 independent experiments.

Proteinase K treated aliquots of similarly fractionated samples were processed identically to determine what proportion of the recovered PEG-phase DNA was protein linked (Table I)

- 32 -

TABLE I

START MATERIAL TREATMENT	SONICATION SECS	ROUNDS	PEG:PO4	YIELD % LOADED
R+ HLWG PK-	30	4	400:700	0.46%
" " PK+	"	"	"	0.00%
R+ HLWG PK-	30	4	400:700	0.70%
" " PK+	"	"	"	0.00%
R+ HLWG PK-	30	4	400:700	1.21%
" " PK+	"	"	"	0.00%
R+ HLWG PK-	30	4	250:700	0.56%
" " PK+	"	"	"	0.00%
R+ HLWG PK-	30	4	250:700	0.36%
" " PK+	"	"	"	0.01%
R+ TRASK PK-	0	5	500:750	7.89%
(DNase)				
" " PK+	"	"	"	0.00%
R+ TRASK PK-	60	5	500:750	11.42%
" " PK+	"	"	"	0.00%
R- TRASK PK-	60	5	500:750	16.38%
" " PK+	"	"	"	0.60%

In order to establish the extent to which PEG-phase yield is dependent on the attached PEG on the protein or the protein itself, PEG-modified and sham treated DNA-protein complexes were compared, also proteinase K treated samples and protein free DNA derived from aliquots of the same complexes. Protein-DNA covalent complexes (largely topoisomerase-bound DNA Trask loc. cit. prepared by the SDS/KCl precipitation method, were either coupled to MPEG using the activated tresyl-MPEG method described below or sham treated with coupling buffer and untresylated MPEG.

This sham treatment resulted in a significantly lower recovery in the PEG phase ($96.3 \pm 4.3\%$ reduced yields mean \pm SEM)

SUBSTITUTE SHEET

- 33 -

of three independent experiments (ranging from 88.8% where 1 round of PO_4 extraction was used, to 100% with 5 rounds of extraction). Where aliquots of the same PEG-modified DNA-protein complexes were subjected to to proteinase K treatment prior to partitioning PEG-phase yields were reduced by $99.7 \pm 0.4\%$ and where proteinase K treatment was followed by phenol chloroform extraction to remove all protein, no DNA was detected in the PEG-phase in all 3 experiments.

Recoveries of DNA from the two phases varies and is circa $38.0 \pm 8.1\%$ for of load for the PEG phase and $49.7 \pm 8.4\%$ for the PO_4 phase using the standard procedure outlined in methods (using desalting columns and ethanol precipitation). Results are means \pm SEM for 6 independent experiments and the difference is statistically significant ($p < 0.01$; paired t-test). This will be important in some applications where the amount of recovered DNA is to be expressed in terms of loaded DNA and not with respect to a similarly handled control (i.e. a control dissolved in and then extracted from the relevant phase(s)).

- 34 -

EXAMPLE 2

USING THE METHOD TO MONITOR LEVELS OF DNA-PROTEIN COMPLEXES
(THIS EXAMPLE IS ALSO RELEVANT TO THE USED OF THE METHOD FOR
THE DETECTION/ASSAY OF DAN BINDING ENZYMES - SEE BELOW)

In order to prepare cells with increasing levels of two different types of DNA-protein complexes with which to evaluate the method we exposed cells to: a) VP-16-213 (etoposide) and b) UV radiation which produces covalent attachment of proteins normally non-covalently bound to DNA.. Figures 5 and 6 show the dose related increment in PEG phase DNA.

VP-16 (15 min exposure) of whole cells was used to increase the level of topoisomerase II complexed to DNA. The results are means \pm SEM of PEG-phase yields for 8 independent experiments, expressed with respect to untreated controls. (Fig. 5).

The amount of DNA complexed to topoisomerase II in the preparation to be fractionated was varied by exposing HL60 cells to increasing concentrations of VP16-213. This drug stabilises the enzyme at the stage where it is covalently bound to DNA (11) and prevents completion of the reaction (the ligation and dissociation steps). Figure 1a shows a dose-related increase in DNA recovery from the PEG phase maximal at 10^{-5} M VP16-213. Phase partitioning was performed as described below (multiple phosphate extractions were not used in these experiments). Results are means \pm SEM of 8 independent experiments and are expressed as a % of the amount of DNA recovered without exposure of cells to VP16-213. In 2/8 experiments the sample was sonicated prior to fractionation. An increment at 10^{-9} M VP16-213 was detected in both the sonicated and 3 of the 6 unsonicated samples, otherwise responses were similar.

This unusual sensitivity of the method is discussed further below, as is the heterogeneity of response (revealed

SUBSTITUTE SHEET

- 35 -

here as a shoulder on the dose response curve and confirmed in additional experiments).

UV cross linking was used to increase the level of protein covalently bound to DNA. The results are means \pm SEM of 3 independent experiments expressed with respect to untreated controls. (Fig. 6).

UV radiation is well known to induce covalent crosslinking between proteins and DNA. This example is relevant to the adaptation of the method for the examination of proteins that bind to DNA non-covalently with the method (also further discussed in an example below).

- 36 -

EXAMPLE 3COMPARISON OF THE SDS/KCL PRECIPITATION METHOD AND THE NEW
TECHNIQUE: IMPROVED RECOVERY OF DNA AND IMPROVED DIFFERENTIAL
RECOVERY BETWEEN DIFFERENTIATING AND UNDIFFERENTIATED CELLS
WHEN USING THE TWO METHODS IN SERIES

We evaluated the performance of the two techniques on differentiating and undifferentiated cells (Table II). Since yield with the new procedure is highly dependent on the average length of the DNA fragments, no definitive yield for the method can be calculated. With 5 seconds sonication the new procedure produces a higher yield than for the Trask procedure. That this occurs despite a much smaller fragment size (circa 20-40 fold) the 5 second sonicated sample indicated that the new method recovers many more DNA fragments. This size differential is illustrated by comparing the Trask material's DNA molecular weight in Figure 9 (Example 6) with 5 second sonication DNA sample in Figure 5 (Example 2). Since each fragment represents one or more DNA topoisomerase II cleavage sites the method is much more efficient with respect to those sites.

- 37 -

TABLE II: COMPARISON OF THE NEW METHOD WITH THE SDS/KCL PRECIPITATION METHOD.

CELL PRE-TREATMENT (HL60 CELLS)	THIS PROCEDURE* (n) Yield, % loaded DNA	TRASK METHOD (n)
	5 SECS SONICATION	
RETINOIC ACID	6.0 ± 3.0 (3)	2.61 ± 0.52 (25)
NONE	6.4 ± 1.6 (3)	2.37 ± 0.48 (25)

* Using 5 seconds sonication and 4 rounds of phosphate extraction (to compensate for the smaller DNA fragment size induced by sonication - see methods).

Although there is independent experimental evidence for DNA/topoisomerase II associations during induction of differentiation, neither technique, used singly, showed significant differences in the amount of DNA recovered from induced and uninduced cells (Table II). This is not surprising, since estimates of the number of DNA breaks (or break clusters) per cell occurring in induced differentiation are low, possibly only 50 per cell and since the modal fragment length with 5 seconds sonication is low, this represents a small proportion of the genome.

We mentioned the theoretical reservations about the SDS/KCl precipitation method for the preparation of DNA/topoisomerase II complexes, that were pointed out Trask et al elsewhere. His method is only efficient for sites where many topoisomerase molecules are attached to a singl

- 38 -

strand of DNA. This may in part explain the differences observed here.

It should be noted that given the possible differences in the distribution of topoisomerase cleavage sites at the replication fork and differentiation-associated break sites, the new method may, since it can partition DNA to which a single topoisomerase molecule is attached, be of advantage for the preparation of differentiation site DNA. This does not of course mean that the Trask procedure cannot be used in series with the new technique to produce a two-step fractionation procedure for differentiation-associated topoisomerase cleavage sites, since even if the efficiency of precipitation is low, differentiation sites with one or few topoisomerase molecules are not likely to be less represented in the precipitate than in the supernatant.

As outlined above, the increased yield with the new method, which could reflect more efficient recovery of DNA associated with single topoisomerase molecules, has implications of the use of the method in applications where very few molecules of protein are attached to DNA (e.g. isolated DNA regulatory sequences are known to contain single topoisomerase II cleavage sites or small clusters of 2-5 sites). Two examples given below corroborate this notion that the new method improves detection of such sites.

1) Experiments where affinity partitioning of very small molecules of DNA was achieved (circa 120-400 bp). Since

- 39 -

previously published DNase protection assays show that fragments of 140 bp are protected at each individual topoisomerase II cleavage site if digestion is carried out in the presence of the enzyme, fragments in this size range should include some that were attached to a single enzyme molecule (see below).

2) The ability of the method to enrich for differentiation-specific protein DNA complexes (see below).

We also explored using the two methods in series (Table III). Here in contrast, in 9 experiments (using 60 sec sonication or 60 sec sonication with additional DNase treatment), there was a significant increase in the proportion of PEG-phase DNA recovered from retinoic acid treated cells when the start material was SDS/DC1 precipitated ("Trask") DNA. This enhanced recovery of DNA from the Trask Material from differentiating cells was dependent on the fragment size selected for the phase-partitioning, since it was observed with small fragments (e.g. at 60 seconds sonication, with or without additional DNase digestion) but was not observed when using 20 seconds sonication. With no sonication, virtually all Trask material (which when prepared avoiding shearing consists mainly of fragments over 20Kbp) carries to the PEG-phase, precluding differential recovery.

TABLE III

CELL PRE-TREATMENT

(HL60 CELLS)

	Yield, % whole genomic DNA		Yield % Trask DNA	
	60 SECS* SONICATIONS	20 SECS SONICATION	60 SECS* SONICATION	20 SECS SONICATION
RETINOIC ACID	0.027±0.015 (8)	0.40±0.14 (10)	1.46±0.33 (9)	11.79±2.34 (10)
NONE	0.020±0.008 (8)	0.57±0.28 (10)	0.52±0.17 (9)	16.09±6.20 (10)
p (paired t-test)			0.029	
	N.S.	N.S.	SIGNIFICANT	N.S.

*With additional DNase digestion in 3 experiments

The differential recovery suggests several possibilities: an increase in the ratio of protein-bound DNA to free in SDS/KCL precipitates from differentiating versus undifferentiated cells (anticipated on the basis of the known actions of DNA topoisomerase II in differentiation) however we cannot exclude: a qualitative change in the types of proteins bound vis a vis their accessible lysyl residues for PEG-modification; or a decrease in the average DNA fragment size of the SDS/KCL precipitates (since with longer fragments it is more difficult to overcome the affinity to the phosphate phase). This latter hypothesis seems unlikely, since, although high resolution electrophoresis of SDS/KCL precipitates has not yet been performed, no dramatic reduction of these fragments below 20Kbp seems to be occurring in differentiated cells.

For increased recovery to occur from the SDS/KCL precipitates without an significant increase in recovery when results are expressed with respect to the start (whole

- 41 -

g nomic) material, does not necessarily indicate that other (non-differentiation associated) protein/DNA covalent interactions may have been lost during induction. The second round recoveries are only a very small fraction of the intermediate recovery and errors in relating the SDS/KCL recovery to the whole genomic DNA could readily have masked any increment.

EXAMPLE 4USING SONICATION TO REDUCE DNA LENGTH AND IMPROVE ENRICHMENT OF THE DNA MOST CLOSELY ASSOCIATED TO THE PROTEIN AND RECOVERY OF COMPLEXES CONTAINING SINGLE PROTEIN-BINDING SITES.

Sonication and other methods to fragment DNA have two influences on the method. Opposing forces act on the DNA-protein complexes. If the DNA attached to a single molecule is long enough it could, via its affinity for the phosphate phase, retain the complex in that phase. Shortening the DNA will reduce the likelihood of this occurring. The second motive for shortening the DNA is if one wishes to recover the DNA at the protein binding site with little contamination by adjacent DNA.

1) The effect of sonication on the partition coefficient of protein free DNA

For the reasons outlined above sonication is sometimes employed to shorten the average length of DNA attached to proteins. It is known however that reducing the length of DNA alters the partition coefficient, reducing the tendency of DNA to remain in the phosphate phase and leading to a more equal distribution between the two phases. The improved enrichment of protein-bound DNA in the PEG phase (enrichment with respect to the DNA-protein binding sites) achieved by sonication will thus be to some extent counteracted by this change since additional protein-free DNA will partition to the PEG phase with increasing sonication (Figs. 2 and 3).

Unlike the PEG/protein/DNA complexes which will have a very high affinity for the PEG phase, the protein free DNA of the PEG phase is extractable by additional rounds of partitioning with fresh phosphate phases or by counter current distribution (i.e. chromatographic separation in a multiple phase partitioning apparatus). The experiment

described in Example 1 and Fig. 4 demonstrates the efficiency of additional rounds of extraction with phosphate phase in removing PEG-phase DNA after sonication.

- 44 -

2) Shortening DNA length of DNA protein complexes reduces the DNA recovery from the PEG-phase

Sonication reduces the average length of the DNA in the DNA/protein preparation and concomitantly reduces the DNA partitioning to the PEG phase. Using the protocol in methods and 5 seconds sonication PEG-phase DNA was reduced to $32 \pm 17\%$ of values in parallel experiments of unsonicated protein-linked DNA (using 4 different types of samples i.e. drug treated and controls).

This modification of the method may be important in achieving detection of complexes where there is only a single isolated protein molecule bound to a section of DNA. It is also potentially useful in applying the method to cloning of DNA at protein binding sites, because sonication removes adjacent DNA.

EXAMPLE 5

ESTABLISHING THAT DNA PROTEIN COMPLEXES WITH A SINGLE ATTACHED TOPOISOMERASE II CAN BE PARTITIONED USING THE METHOD

Figure 7 shows an example of affinity partitioning of small PEG/protein/DNA complexes. DNA/protein complexes were sonicated for 60 seconds to fragment the DNA. Topoisomerase II is known to protect DNA fragments of circa 140 base pairs in DNase protection assays. Thus fragments of this size are unlikely to have more than single molecule of enzyme attached to the DNA.

This experiment shows small fragments (mainly between molecular weight markers of 564 and 125 bp) recovered from the PEG-phase after partitioning. That these fragments are not due simply to the reduced tendency of small DNA molecules to remain in the phosphate phase (the problem mentioned above) is demonstrated by a control sample which was treated with proteinase K before the coupling step (coupling PEG to the protein/DNA complexes).

- 45 -

Because of the anticipated very small DNA fragment sizes not only were additional rounds of phosphate extraction used here but the ratio of volumes of the two phases was altered to PEG:PO₄ 250:750ul (3 changes of 750ml PO₄ were used).

- 46 -

This experiment is relevant to the claim that the new technique should improve the detection of differentiation-specific topoisomerase II DNA complexes, some of which we know to be relatively isolated in the genome (see Example 7 below).

EXAMPLE 6

USING NUCLEASES TO REDUCE DNA LENGTH AND IMPROVE ENRICHMENT OF THE DNA MOST CLOSELY ASSOCIATED TO THE PROTEIN AND RECOVERY OF COMPLEXES CONTAINING SINGLE PROTEIN-BINDING SITES.

We have also established that nucleases (e.g. DNase and restriction endonuclease) can be used to reduce DNA size of the DNA/protein complexes. DNase digestion of DNA protein complexes prepared by the Trask method, using the protocol described in methods, after 10-20 minutes produces fragments comparable to 60 seconds sonication (Figure 8). In a similar preparation the protein "protects" a low molecular weight band of DNA from digestion (inset in Fig 8): lanes 1 & 2 = no DNase; lanes 4-6 = 1,5,10 min DNase digestion after proteinase K (the DNA is unprotected and largely digested); lane 7 = HindIII/EcoRI DNA; lanes 8-10 = 1,5,10 min DNase digestion of protein/DNA complexes (a low molecular weight band of DNA circa 130 bp is protected). This method can also therefore be used to reduce DNA size for partitioning either before the translation step (Figure 9), or after the first round of partitioning (Figure 10) before subsequent partitioning or DNA cloning. The protein K digested control in Fig 9 demonstrates that the recovered low molecular weight DNA is the result of affinity partitioning since none is recovered if the PEG-protein is first removed from the DNA by proteinase K digestion.

Since the attached protein "protects" the attached DNA from digestion by DNase, the method can be used to prepare samples enriched for such fragments. This has

- 47 -

applications in the analysis of DNA protein binding sites using the DNase protection principle.

As might be expected on the basis of the small fragment size produced by 60 second sonication of DNA/protein complexes, an additional partitioning step after DNaseI only produced a modest reduction in yield of DNA from the PEG phase. Result show yields from Trask preparations from retinoic acid induced and uninduced HL60 cells and are means \pm SEM for three experiments using both DNase and 60 seconds soication, versus the mean \pm SEM fpr 5 experiments using sonication alone.

PEG-Phase yields from Trask preparations comparing three experiments using both DNase and 60 seconds sonication, versus 4 experiments using sonication alone are shown in Fig. 10. A modest reduction in yield is seen with material from both retinoic acid and uninduced HL60 cells.

- 48 -

EXAMPLE 7USING THE METHODS TO ENRICH FOR DIFFERENTIATION-SPECIFIC
DNA/PROTEIN COMPLEXES.a) Demonstration of recovery of quantitatively different
amounts of DNA from SDS/KCL precipitates from differentiating
cells

We know from previous studies that retinoic acid induces DNA topoisomerase II to perform DNA breakage-reunion reactions shortly after the induction of differentiation. This can be demonstrated in several ways (e.g. nucleoid sedimentation, the alkaline filter elution technique and the fluorescent alkaline DNA unwinding - FADU- technique) and is inhibited by DNA topoisomerase II inhibitors (13). None of these methods is however suitable for the purification of these complexes. The Trask procedure as mentioned above, is likely on theoretical grounds to be inefficient for isolated protein molecules attached to DNA. It also does not in practice recover greater amounts of DNA/protein complexes from retinoic acid treated cells than from controls (see Examples 3 above). The Trask technique is unlikely therefore to be providing a significant enrichment of this type of DNA/protein complex vis a vis other complexes (the technique is known to be highly efficient for DNA/topoisomerase II complexes from DNA replication forks).

In order to examine whether the new technique can enrich for and/or recover sufficient quantities of the retinoic acid induced complexes for their presence to be detected, we employed the two "short fragment" modifications described above: either 60 seconds sonication, or sonication plus an additional round of partitioning after DNase treatment. The start material in each case was a DNA/protein complex preparation precipitated by the Trask method.

As Figure 8 shows, in 7/9 experiments a higher proportion of the start material was recovered from the

- 49 -

retinoic acid treated material. This difference is statistically significant ($p = 0.029$; paired t-test).

Differential yields from Trask material when prepared from differentiating and undifferentiated HL60 cells are shown in Figure 11.

b) Demonstration that specific DNA sequences with differentiation-associated attachment to protein are enriched by the procedure

To test whether differentiation involves specific DNA sequences, we obtained highly purified Topo II-associated DNA from RA induced differentiating cells, labelled and hybridised this DNA to DNA enriched for, or depleted of, Topo II-bound DNA from both undifferentiated HL60 cells and cells induced to differentiate by RA or phorbol ester.

Differential hybridisation demonstrated that the isolated DNA is enriched for specific sequences. Differential hybridisation was also seen with myc and fos probes suggesting that Topo II cleavage occurs near these genes. This is, as far as we are aware, the first demonstration that Topo II interacts at specific (or limited) sites in the genome during the induction of differentiation.

It is important to note that although enhanced recovery is seen when topoisomerase II is arrested at the covalent complex stage by VP-16 (etoposide), this agent is not used in the following experiments since it is known to influence cleavage site specificity.

Since opposing forces act in the phase partitioning system (longer DNA fragments oppose affinity partitioning) theoretically the maximum efficiency of recovery of cleavage site DNA will occur with the lowest possible fragment length. To this end we have used DNase digestion to remove protruding DNA (topoisomerase are known to protect circa 140bp of DNA as

- 50 -

they act). This experimental detail is particularly important for the recovery of differentiation specific breaks sites since it will reduce the presence of contaminating repetitive sequences.

DNase digestion of DNA/Topo II complexes prepared by the Trask method for 1, 2 and 5 minutes demonstrates a clear protected band of DNA and this material can be affinity partitioned (see Examples above).

Since we have demonstrated that affinity partitioning (as opposed to the Trask method) can recover DNA attached to a single topoisomerase molecule, the new method has theoretical advantages for the preparation of highly enriched DNA from differentiation cleavage sites (whereas the Trask technique is most efficient for replication fork DNA).

To test whether the Topo II cleavage sites are at specific sites in the genome we prepared sets of 4 different DNA samples:-

- 1) Topo-associated DNA from differentiating cells
- 2) Topo-associated DNA from undifferentiated cells
- 3) DNA depleted of Topo cleavage sites from differentiating cells
- 4) DNA depleted of Topo cleavage sites from undifferentiating cells

Using dot blot hybridisation to DNase protected Topo-associated DNA from differentiated cells the anticipated patterns of hybridisation (if specific DNA sequence become associated with topoisomerase or other proteins during differentiation and are thus recovered by the method) are as follows:-

- 1) +++ (enriched relative to undifferentiated control)
- 2) ++
- 3) + (depleted relative to undifferentiated control)
- 4) ++

Differential hybridisation shows that specific sequences

- 51 -

b come protein-bound during retinoic acid induced granulocytic differentiation (Figure 12).

Differential hybridisation is also observed when the filter DNA is prepared from phorbol ester induced cells (Figure 13). Thus some sites at least are shared between monocytic and granulocytic differentiation. This results indicates that the DNA sequences recovered by this method may have broader applicability than just defining differentiation events in one specific cell lineage and inducer system.

c) Demonstration that the differential hybridisation is reproducible

Fig. 14 shows that similar differential hybridisation is achieved with independently prepared filter and probe samples (2 probe and 6 filter samples). PMA hybridisation also showed reproducible differential hybridisation. In 10/10 hybridisations there is a more intense signal from the R+ than R- DNA as shown in Figure 14.

d) Demonstration that genes with known changes in expression during haemopoietic cell differentiation are fractionated by the method (and demonstration that this is due to sequences in or around the gene undergoing topoisomerase association)

This differential hybridisation is also observed when the filter are probed with both myc and fos probes.

The success in enriching for two genes whose expression is known to be modulated during haemopoietic cell differentiation (c-myc and c-fos) suggests that the new technique provides a strategy to search for hitherto unknown differentiation genes. Break-site DNA is currently being cloned for identification of "differentiation genes" and putative regulatory sites.

These findings indicate that differentiation associated Topo II cleavage reactions (and/or other DNA protein linking

- 52 -

events) take place in or near the myc and fos genes during induced granulocytic differentiation. Since differential hybridisation was lost for myc when novobiosin was added (see Figure 16 other DNA protein linking events seem unlikely. Scanning densitometer traces of Fig. 16 show differential hybridisation to the myc probe by R+ and R- DNA is lost when R+ cells are pretreated with novobiocin (an inhibitor of Topo II); lines, A, B and C respectively.

Since the differential hybridisation observed between DNA from differentiating and undifferentiated cells was abolished by novobiocin a known inhibitor of Topo II. This suggests that covalent attachment to this enzyme was, although not necessarily responsible for all DNA recovered, responsible for the recovery of DNA influencing the differential hybridisation.

Quantitative analysis of the amount of DNA recovered from differentiating and undifferentiated cells suggests that the DNA responsible for the differential hybridisation may be particularly sensitive to the inhibitory action of novobiocin (Figs. 17 and 18).

Novobiocin induced a modest reduction in the recovery of "Trask" DNA, but only in differentiating cells, as shown in Fig. 17.

Novobiocin produced no significant decrease in the amount of DNA recovered by the combined Trask/new procedure, neither in cells induced with retinoic acid nor in undifferentiated cells as shown in Figure 18.

e) Demonstrations that DNA flanking stably expressed (differentiation unmodulated) genes is not enriched by the method.

Similar hybridisation experiments to those for myc and fos were performed using a tubulin gene probe (Cambridge BioSciences). Figures 19 and 20 show that with neither retinoic acid nor phorbol ester induced cells is there

differentiatial hybridisation to the tubulin probe with PEG-phase recovered protein associated DNA.

Lack of differntial hybridisation with R+ and R- DNA (under similar conditions to those used for myc and fos) when the gene probe is tubulin is shown in Fig. 19.

Lack of differential hybridisation with PMA+ and PMA- DNA (under similar conditions to those used for myc and fos) when the gene probe is tubulin is shown in Fig. 20.

- 54 -

EXAMPLE 8USING THE METHOD TO PREPARE DNA FOR CLONING AND
DEMONSTRATIONS THAT SOME CLONES SHOW DIFFERENTIAL
HYBRIDISATION TO DIFFERENTIATION-ASSOCIATED TOPOISOMERASE
CLEAVAGE SITE DNA

Since one use of the method is to prepare specific DNA sequences for cloning we have cloned DNA from retinoic acid induced DNA/protein complexes (prepared as described above with either sonication or DNase digestion) to demonstrate that the method produces DNA suitable for cloning procedures.

Two different cloning techniques were evaluated, but in principal any standard technique could be used.

A) The homopolymer tailing technique was used to introduce the prepared DNA fragments into dCTP tailed Plasmid (pUC8). This method was selected because no differential handling of topoisomerase cleavage sites, sheared ends or restriction sites is involved (we wanted to avoid differences in efficiency of processing fragments with and without the topoisomerase staggered cleavage sites). Transformation followed by growth of plasmid in, and recovery from DH5 E. coli, demonstrated that recombinant plasmids were produced. However the efficiency of this method was relatively low.

Scanning densitometry of agarose gel electrophoresis of plasmids from which DNA inserts have been cleaved by EcoRI/HindIII digestion as shown in Fig. 21.

B) The second strategy was to infill the overhang created by the topoisomerase cleavage reaction using the Klenow polymerase and to attach Sal I linkers. After digestion with EcoRI and SAL I, the DNA was ligated into pUC18, similarly digested, and transformed into E. coli DH5 cells.

Clones were screened for differential hybridisation to R+ R- and whole genomic probes (i.e. using a similar

strategy to that used above), to determine DNA inserts which represent DNA sequences preferentially associating with topoisomerase during the induction of differentiation. A sample of 23 of the first 298 clones obtained shown in Figures 22 and 23.

The combined hybridisation signal (absorbance per mm²) for three hybridisations to measured amounts of DNA from clones is shown in Fig. 22 R+ and R- probes were as for the differential hybridisation experiments above, WG was labelled whole genomic (unfractionated) HL60 cell DNA.

The hybridisation signals (absorbance per mm²) for three hybridisations, given in fig. 22 are expressed as a proportion of the combined hybridisation signal in Fig. 23.

These results demonstrate not only that the material can be cloned, but that a proportion of resulting clones have specific DNA sequences that become protein associated as a result of inducing differentiation. Since topoisomerase modulates gene expression by a mechanism requiring it to act in the vicinity of the affected gene, these DNA fragments will be in or near differentiation genes modulated by topoisomerase II.

EXAMPLE 9USING THE METHOD FOR THE DETECTION/ASSAY OF TOPOISOMERASE INHIBITORS

As implied by the dose response curves above in Example 2, the method can be used to quantitate the complexing of topoisomerase to DNA induced or inhibited by different classes of topoisomerase II inhibitors.

The method is very sensitive for the detection of VP-16, detecting a $326 \pm 100.25\%$ increase in PEG phase DNA at 10^{-9} M VP16 (means \pm SEM of 10 experiments using either undifferentiated or differentiation induced HL60. An increment (maximum 950%) was detected in 7/10 experiments. Increments in PEG phase DNA in the majority of samples at 10^{-9} VP16-213 as shown in Fig. 24.

Uninduced cells were exposed to VP16 for 15 min. Induced cells were exposed to retinoic acid (10^{-6} M) for 70 minutes and simultaneously to the VP16. As mentioned in Example 2 above sonication influenced the proportion of samples showing an increment and thus this and other facets of the method may have to be optimised to yield the most sensitive assay for any individual system.

This result indicates that the new method may be much more sensitive to the action of inhibitors than other assays which usually detect VP16 only at the range 10^{-7} to 10^{-5} M.

This sensitivity is not restricted to the epipodophyllotoxin type topoisomerase II inhibitors and similar results can be obtained with intercalators.

The effect of m-AMSA, an intercalator, on PEG phase yield is shown in Fig. 25. Since the inactive analogue o-AMSA (which does not significantly inhibit topoisomerase II) influenced phase partitioning results, yields were corrected with respect to an o-AMSA control.

EXAMPLE 10

- 57 -

USE OF THE METHOD FOR THE DETECTION OF HETEROGENEITY IN
RESPONSE TO INHIBITORS

After induction of cells by retinoic acid for 70 minutes and concomitant exposure to VP16 (the latter at the doses shown), the enhancement of PEG phase yield is clearly biphasic. The results in Figures 24 and 25 shown two independent experiments. Although only preliminary data, a similar "shouldering" effect is seen in unstimulated cells. This observation may be related to the observation in cell systems that VP-16 inhibits differentiation at 10^9 M whereas it inhibits proliferation only at 10^7 M and above, indicating functional heterogeneity of the enzyme involved in the two processes.

The method thus provides a method for discriminating topoisomerases with inherent or imposed different susceptibilities to inhibitor.

The effect of VP-16 on PEG phase yield after retinoic acid treatment is shown in Fig. 26.

EXAMPLE 11

USING THE METHOD TO EXAMINE NON-COVALENT
ASSOCIATIONS/DISSOCIATIONS BETWEEN PROTEINS AND DNA

If a crosslinking step is used, in theory, changes in the association/dissociation of proteins that do not normally covalently link themselves to DNA may be assessed by the method. We have previously established that during retinoic acid induction of haemopoietic cell differentiation there is an increase in the amount of protein associated with DNA. To demonstrate whether the new technique could detect this, cells were exposed to retinoic acid for the periods shown and were then subject to UV illumination to crosslink protein to DNA. Although cross linking is a stochastic process, any net increase in protein association should be revealed in an increase in the crosslinking of proteins to DNA. Since HL60 are notoriously susceptible to stress and to shock-induced

differentiation, sham treated (handling) controls were used and results for each time point were expressed with respect to these controls.

As figure 27 shows, there is a significant increase in PEG-phase protein associated DNA peaking at 70min exposure to retinoids. Since a similar increment was not observed without a UV crosslinking step (cf. Table II above, where there is no significant difference in PEG phase DNA after 70 min retinoic acid treatment), this increase is concluded to be due to cross linking of non-covalently bound proteins which do not contribute significantly to the PEG-phase yield under the standard conditions.

REFERENCES

- 1) Walter H, Brooks DE, & Fisher D. (eds.) Partitioning in aqueous two-phase systems, theory, methods, uses and applications in biotechnology. Academic Press, Orlando 1985.
- 2) Albertson P-A. Partition of cell particles and macromolecules. 3rd Edition, John Wiley and Son, New York, 1986.
- 3) Delgado C, Francis GE, Fisher D. Coupling of PEG to proteins by activation with tresyl chloride. Applications in immunoaffinity cell partitioning. In: Advances in separations using aqueous phases in cell biology and botechnology. Eds. Fisher D and Sutherland IA. Plenum Press 1988, in press (Proceedings of the 5th International Conference of Phase Partitioning, Oxford Aug. 1987).
- 4) Jordan CF, Lerman LS, Venable JH. Structure and circular Dichroism of DNA in concentrated polymer solutions. Nature New biology 236:67-70; 1972. 5) Maniatis T, Venable JH, Lerman LS. The structure of psi-DNA. J. Mol Biol. 84:37-64;1974.
- 6) Zwelling LA, Michaels S, Erickson LC, Ungerleider RS, Nichols M, Kohn KW: Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4' -(9-acridinylamino)methanesulphon-m-aniside and adrimycin. Biochemistry 20:6553-6563,198
- 7) Trask DK, DiDonato JA, Muller MT. Rapid detection and isolation of covalent DNA/protein complexes: application to topoisomerase I and II. EMBO J 3:671-676,1984

- 8) Francis G.E. & Pinsky C. Growth and differentiation control. In: Cancer Chemotherapy and Biological Response Modifiers annual 9. Eds. H.M. Pinedo, B.A. Chabner and D.L. Longo. Elsevier Science Publishers B.V. 1987 (reprint enclosed).
- 9) Darby MK, Herrera RE, Vosberg H-P, Nordheim A: DNA topoisomerase cleaves at specific sites in the 5' flanking region of c-fos proto-oncogenes in vitro. EMBO J. 9:2257-2265, 1986.
- 10) Muller M Partitioning of nucleic acids. pp 227-266 in: Walter H, Brooks DE, & Fisher D. (eds.) Partitioning in aqueous two-phase systems, theory, methods, uses and applications in biotechnology. Academic Press, New York 1985.
- 11) Chen GL, Yang L, Rowe TC, Halligan BD, Tewey KM, Liu LF. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. J Biol Chem 259:13560-13566, 1984.
- 12) Liu LF, Wang JC. DNA-gyrase complex: the wrapping of the DNA duplex outside the enzyme. Cell 15:979-984;1978.
- 13) Francis G.E., Berney JJ, North PS, Khan Z, Wilson EL, Jacobs P, Ali M. Evidence for the involvement of DNA topoisomerase II in neutrophil granulocyte differentiation. Leukaemia 1:653-659, 1987.
- 14) Francis GE, Khan Z, Gray DA, Wing MA, Berney JJ, Guimaraes JETE, Hoffbrand AV. DNA strand breakage and ADP ribosyl transferase mediated DNA ligation during stimulation of human bone marrow cells by granulocyte-macrophage colony stimulating activity. Leuk Res 8:407-415, 1984.

- 61 -

15) Khan Z., Francis G.E. Contrasting patterns of DNA strand breakage and ADP-ribosylation dependent DNA ligation during granulocyte and monocyte differentiation Blood 69:1114-1119; 1987.

16) Weniger P: An improved method to detect small amounts of radiation damage in DNA of eukaryotic cells. Int J Radiat Biol 36:197-199, 1979.

17) Zwelling LA, Michaels S, Erickson LC, Ungerleider RS, Nichols M, Kohn KW: Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4'-(9-acridinylamino)methanesulphon-m-aniside and adrimycin. Biochemistry 20:6553-6563, 1981

18) T. Maniatis et al, Molecular cloning: A laboratory manual. Cold Spring Harbour 1982.

- 62 -

CLAIMS

1. A process for separating covalent DNA/protein complexes from non-covalent DNA/protein complexes and unbound DNA comprising the steps of:

- (i) treating the DNA/protein complexes with a reactive derivative of polyethyleneglycol (PEG) and
- (ii) subjecting the product of step (i) to phase partition between an aqueous PEG solution phase and an aqueous phosphate solution phase.

2. A process according to claim 1 comprising the further step of:

- (iii) recovering covalently bound DNA/protein complexes from the aqueous PEG phase.

3. A process according to claim 1 or claim 2 wherein the reactive derivative of PEG is 2,2,2-trifluoroethanesulphonyl monomethoxy polyethyleneglycol (TMPEG).

4. A process according to any one of claims 1 to 3 wherein the covalently bound DNA/protein complex is a DNA/topoisomerase complex.

5. A process according to claim 4 wherein the DNA topoisomerase is DNA topoisomerase I and/or II.

6. A process for purifying DNA comprising or associated with an inducible gene wherein induction of transcriptional activity is accompanied by the action of one of more enzymes forming covalent intermediates with DNA comprising separating a covalent DNA/enzyme complex by a process according to any one of claims 1 to 5.

7. A process for purifying DNA comprising a protein binding site comprising, where necessary, effecting covalent binding of the protein to the DNA at the protein binding site, and separating a covalent DNA/protein complex by a process according to any one of claims 1 to 5.

- 63 -

8. A process for assaying DNA topoisomerase activity comprising separation of DNA/topoisomerase complexes by a process according to claim 4 or claim 5.

9. A process for assaying DNA topoisomerase cleavage-site specificity comprising separating covalent DNA/topoisomerase complexes by a process according to claim 4 or claim 5.

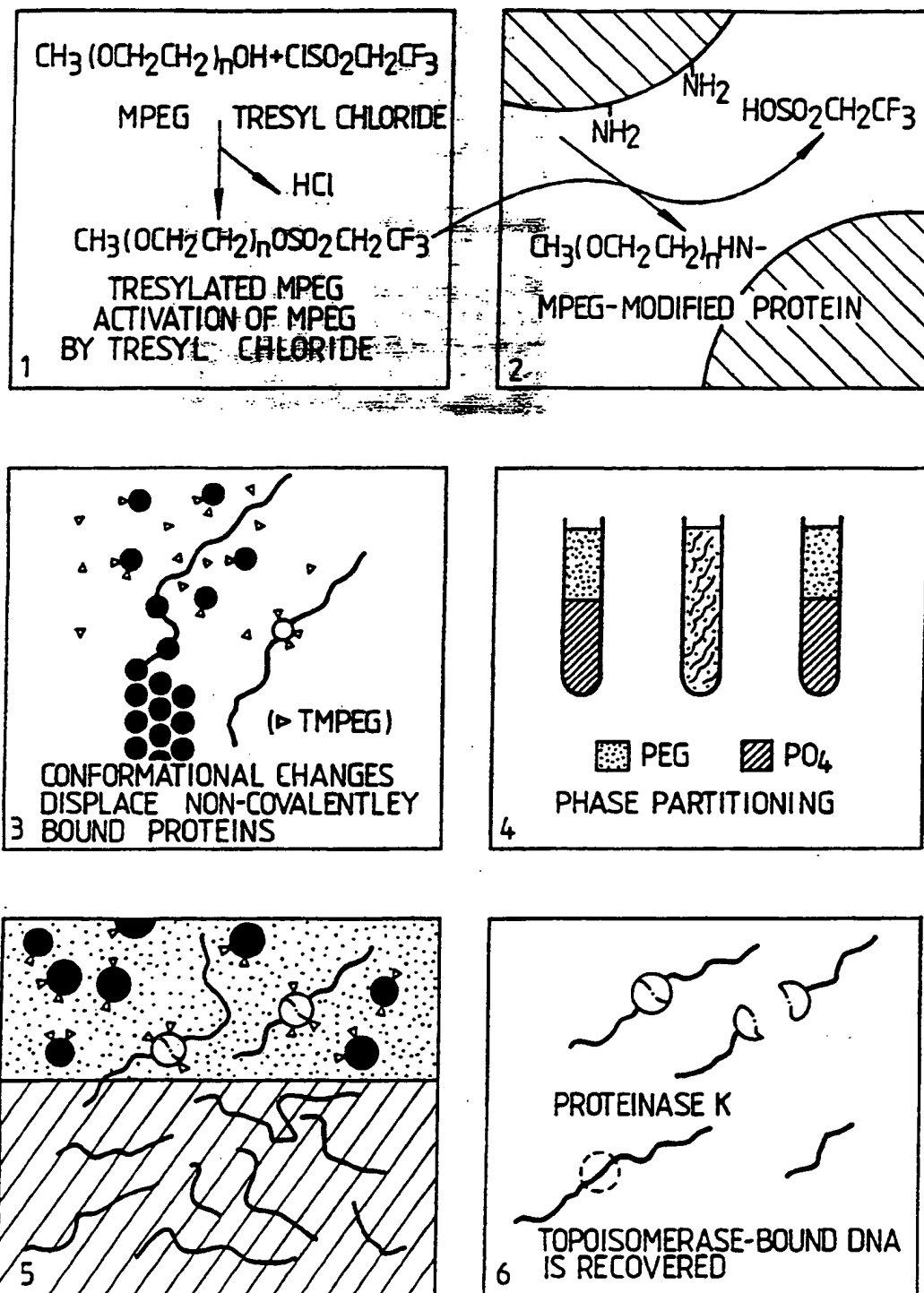
10. A process for producing DNA comprising a protein binding site which process comprises forming a covalent complex of a DNA fragment comprising the protein binding site and a protein which binds to the site, where necessary reducing the length of the DNA fragment by digestion using a DNAase enzyme and separating the resultant DNA/protein complexes by a process according to any one of claims 1 to 5.

11. A process for assaying DNA-protein cross-linking agents comprising separating covalent DNA/protein complexes by a process according to any one of claims 1 to 5.

12. A process for assaying an inhibitor of a nicking/closing enzyme comprising separating covalent DNA/enzyme complexes by a process according to any one of claims 1 to 5.

1/18

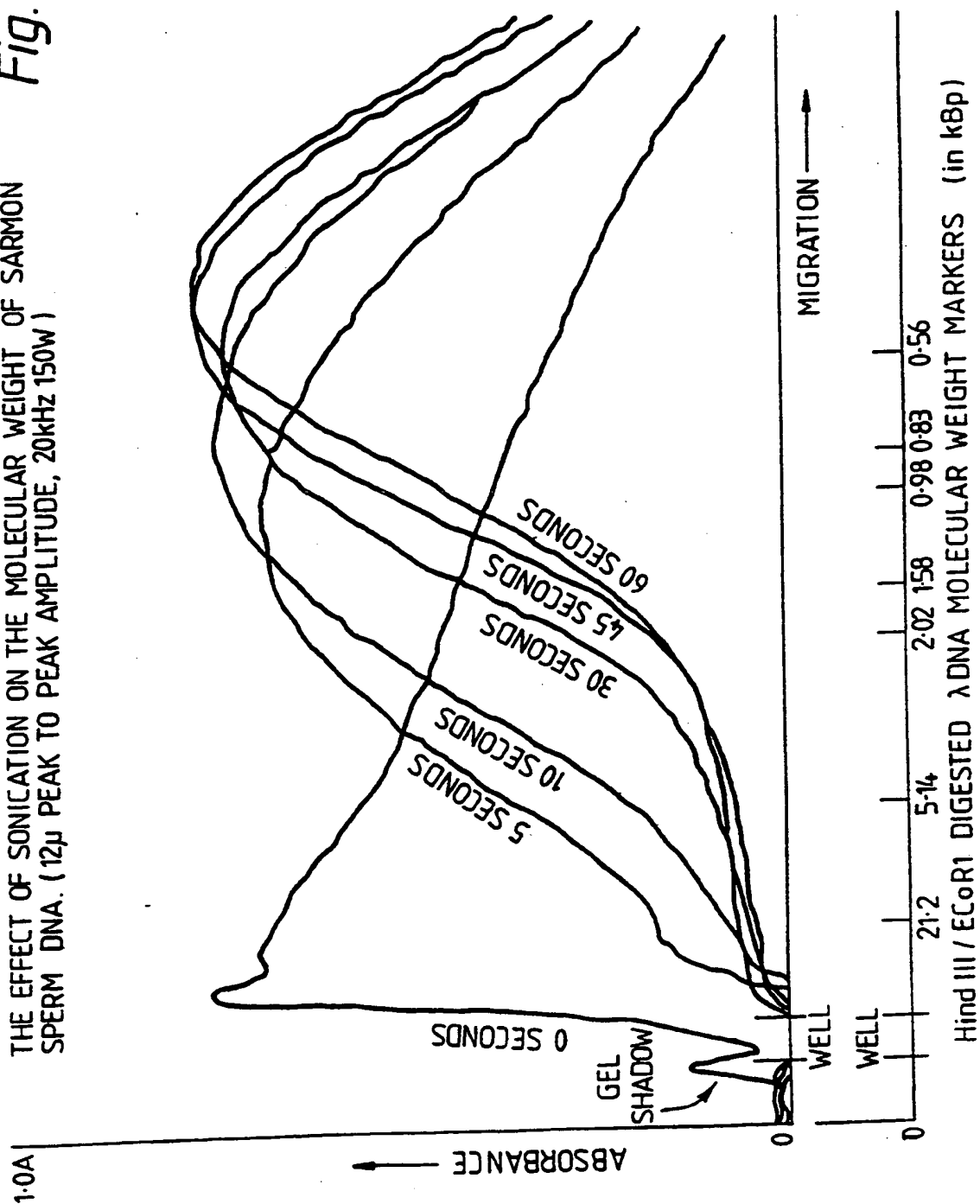
Fig. 1.



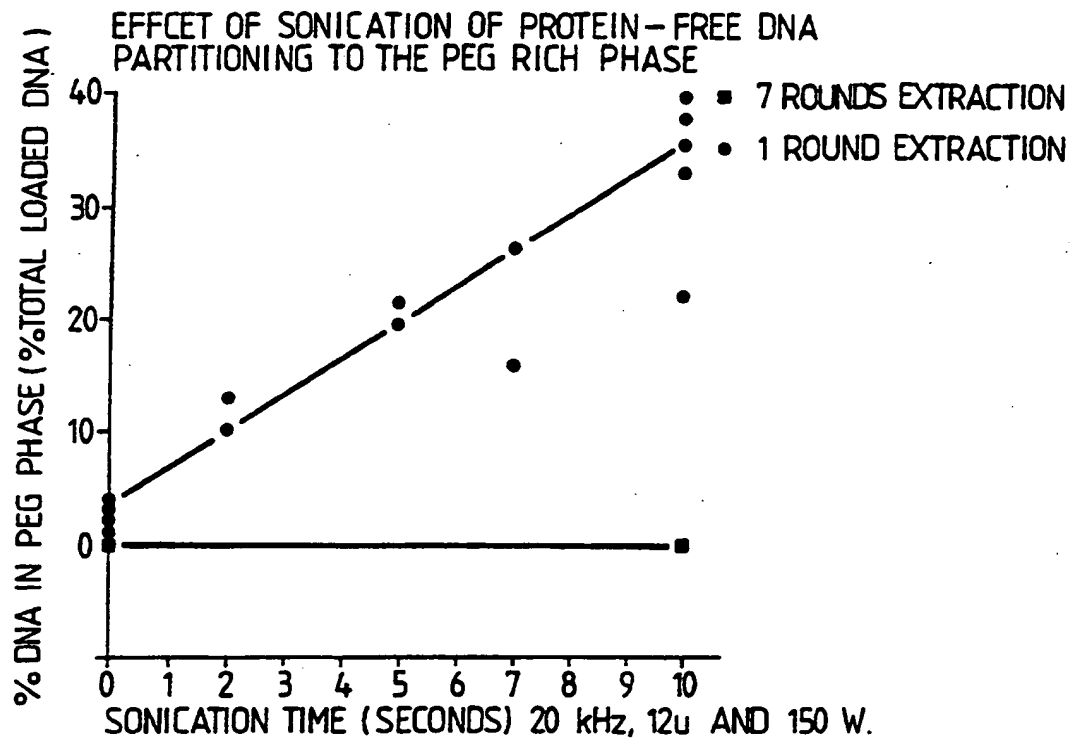
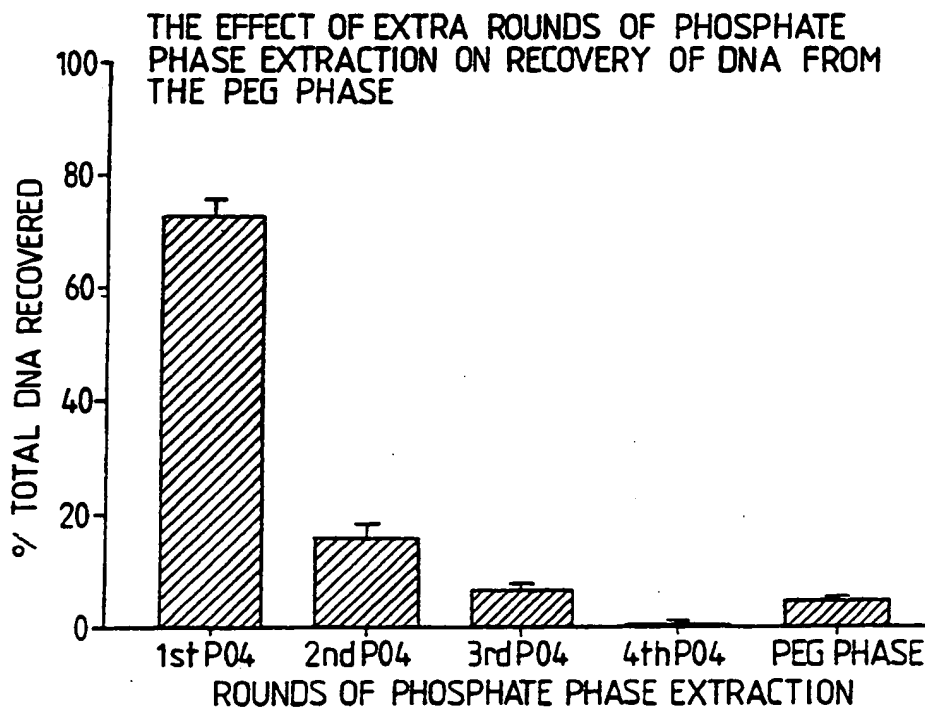
2/18

Fig. 2.

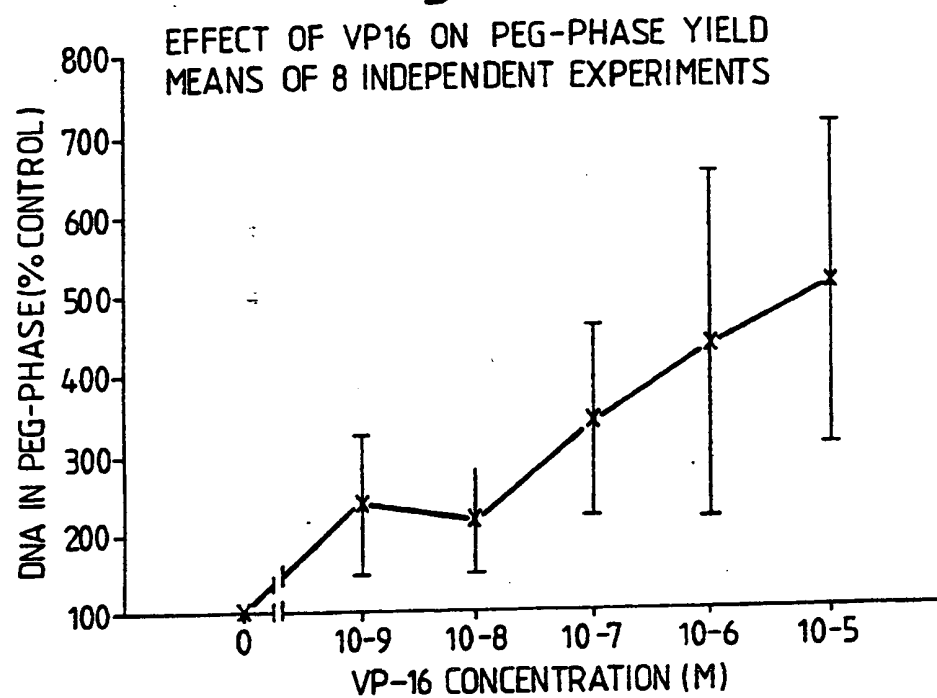
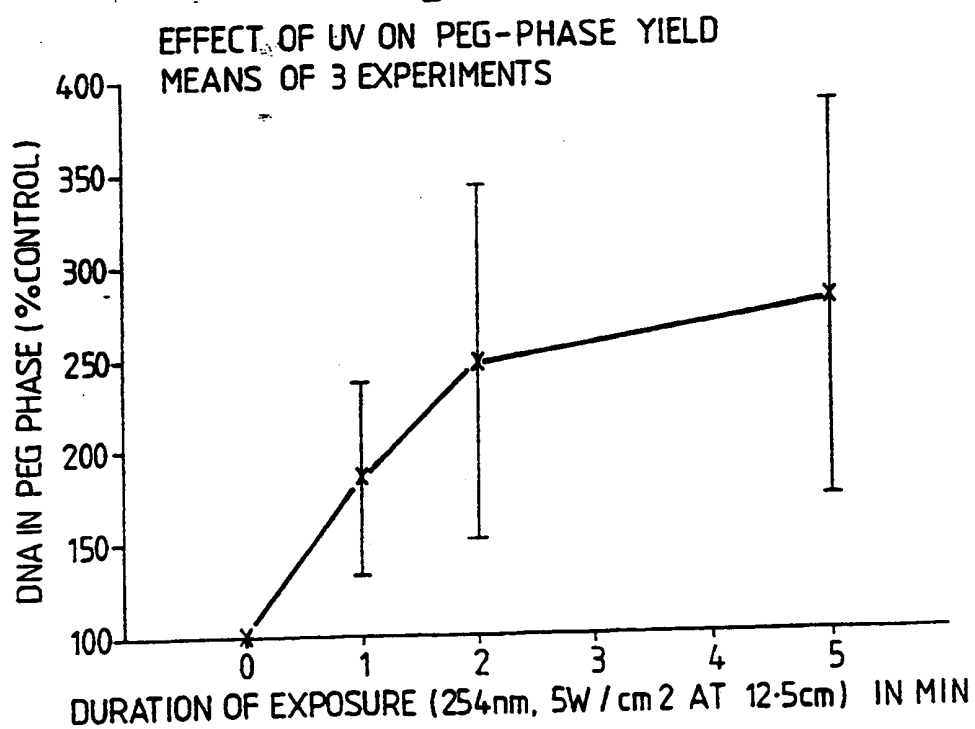
THE EFFECT OF SONICATION ON THE MOLECULAR WEIGHT OF SARMON
SPERM DNA. (12 μ PEAK TO PEAK AMPLITUDE, 20kHz 150w)



3/18

Fig. 3.*Fig. 4.*

4/18

Fig. 5.*Fig. 6.*

5/18

Fig. 7.

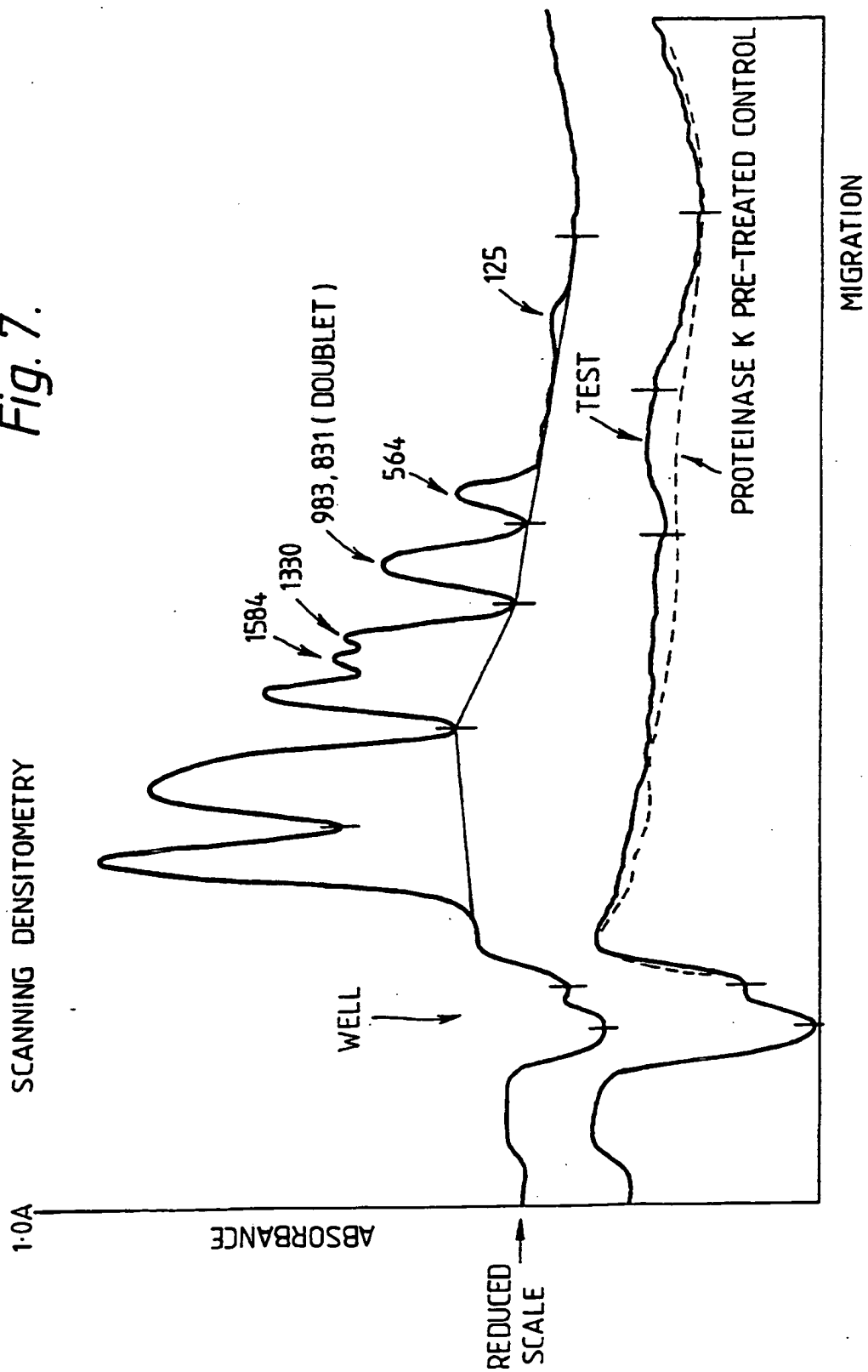
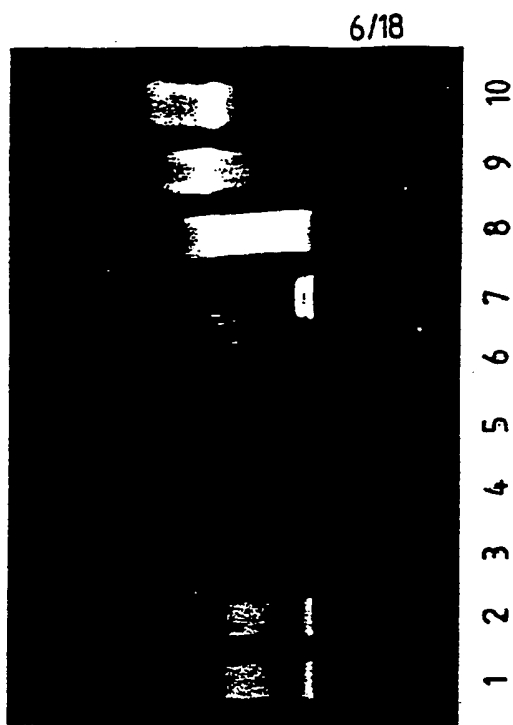
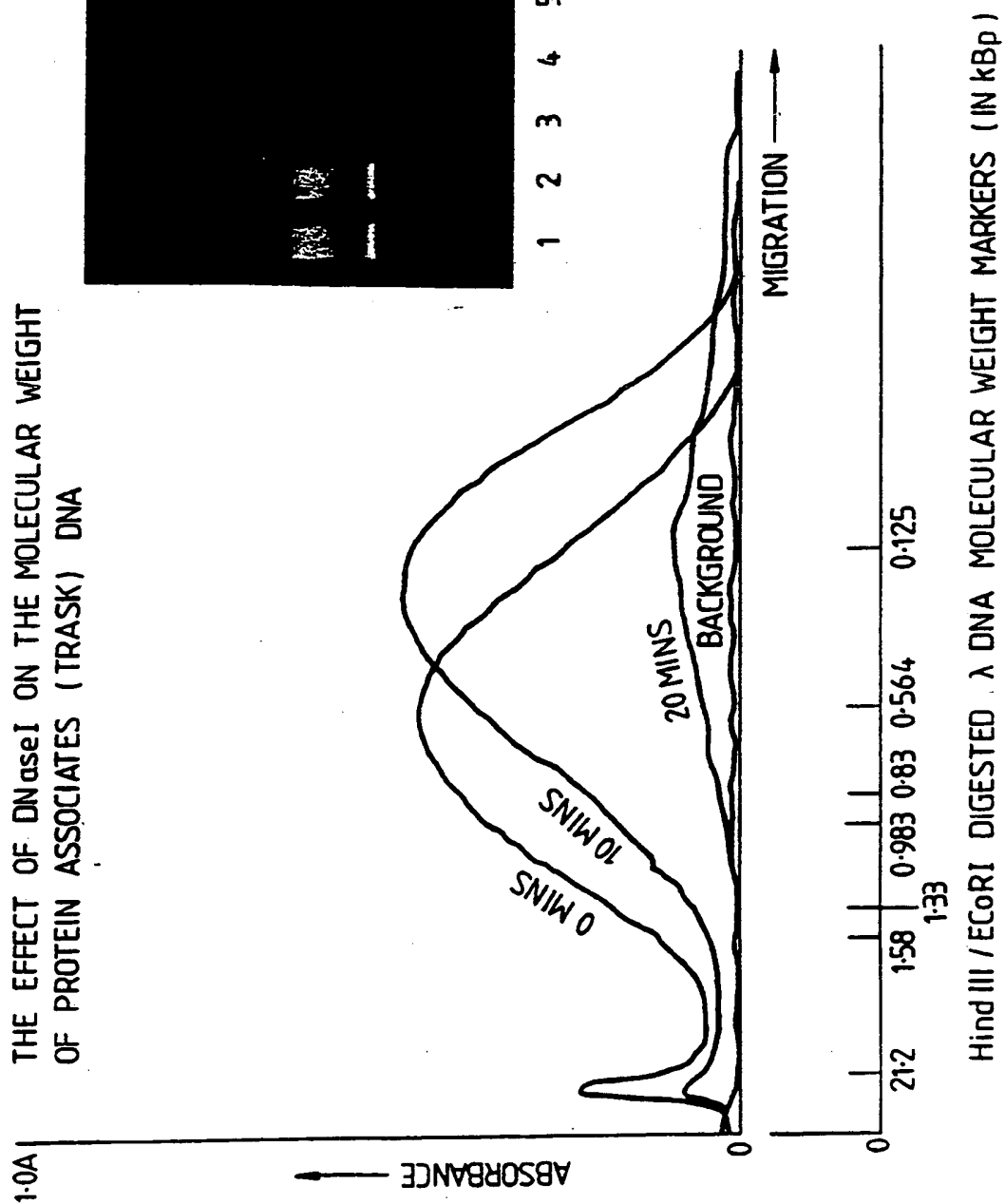


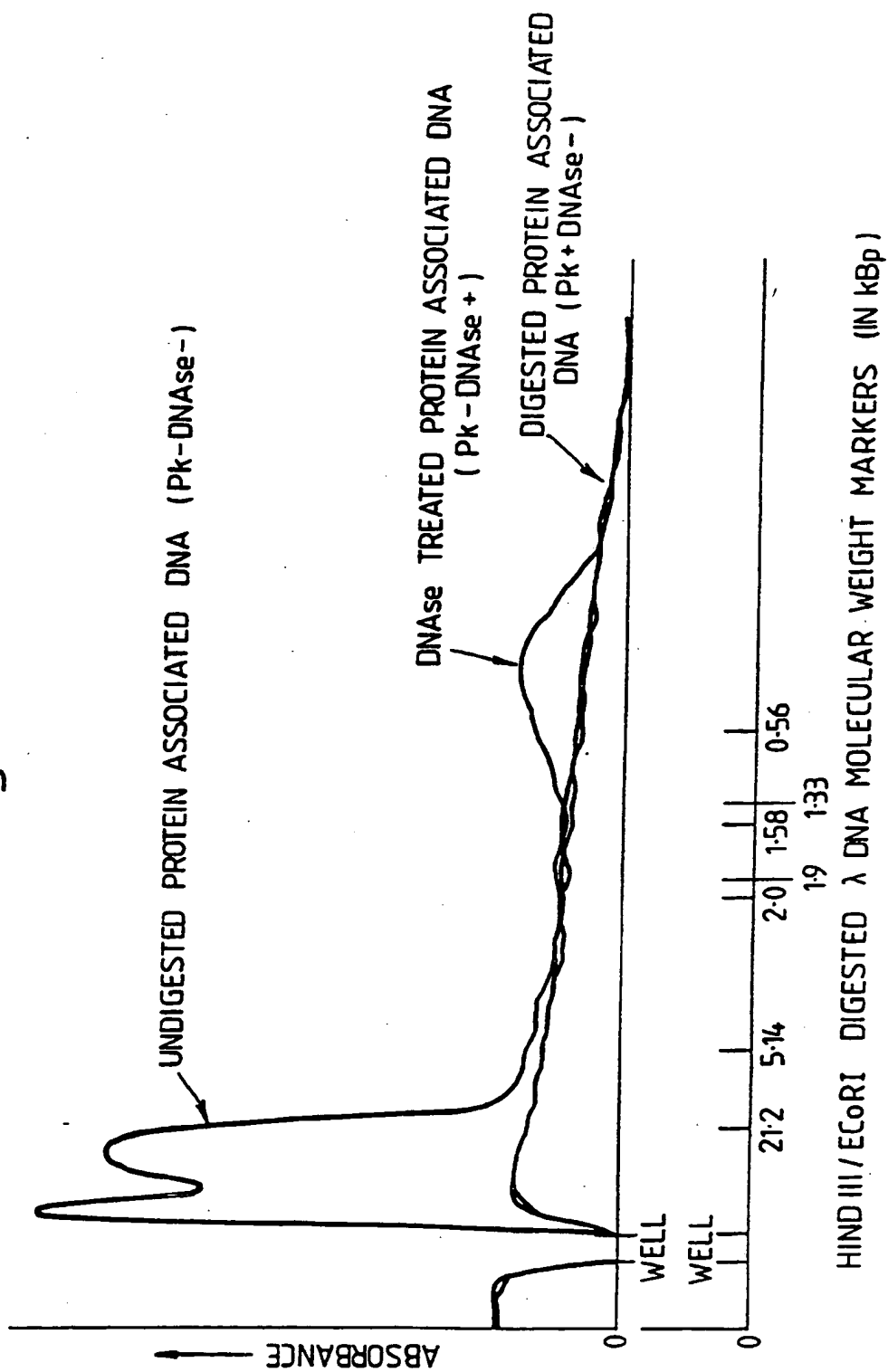
Fig. 8.

THE EFFECT OF DNaseI ON THE MOLECULAR WEIGHT
OF PROTEIN ASSOCIATES (TRASK) DNA

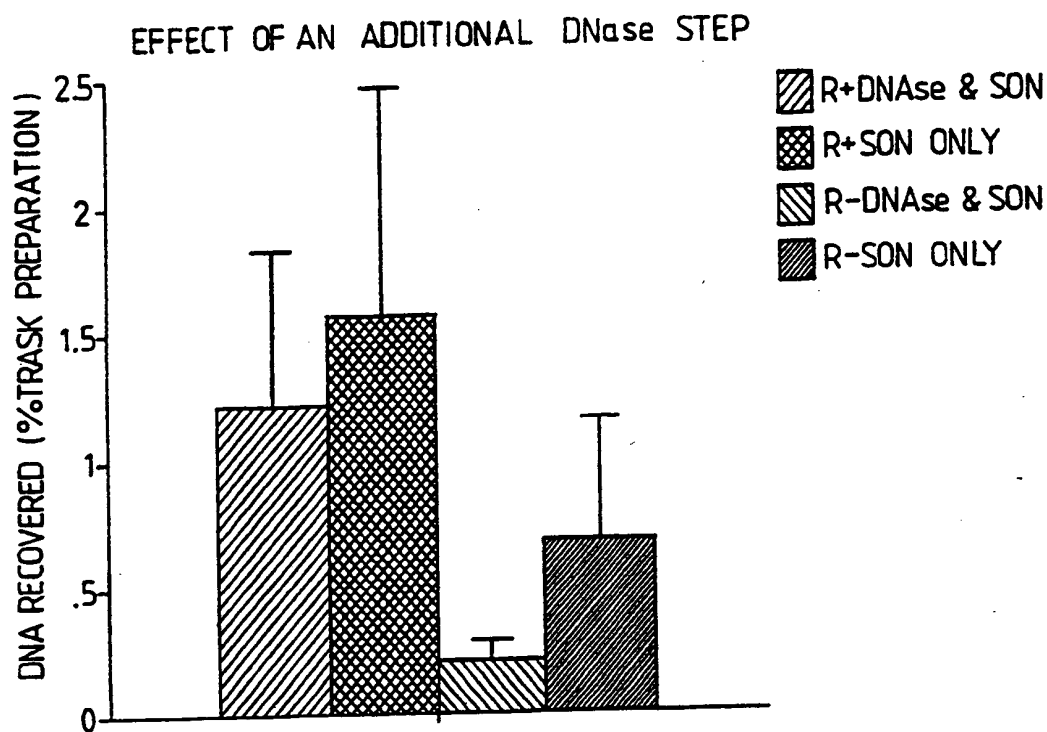
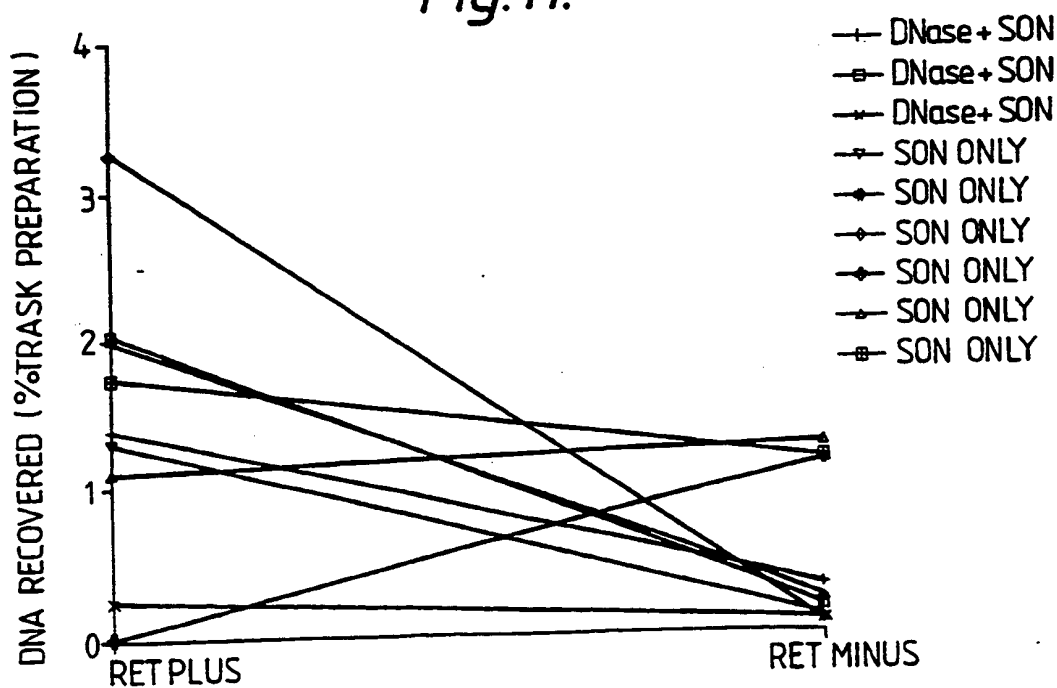


7/18

Fig. 9.

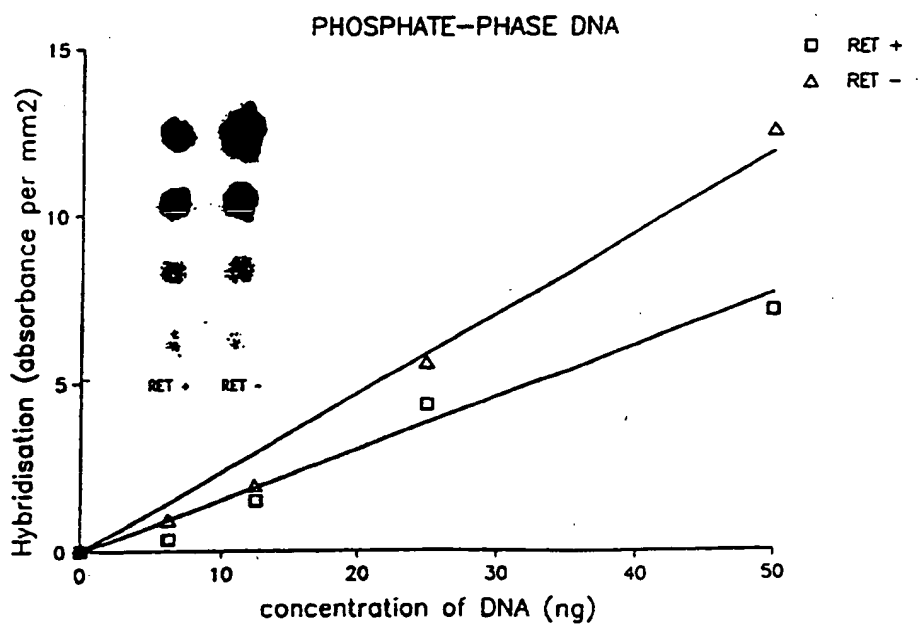
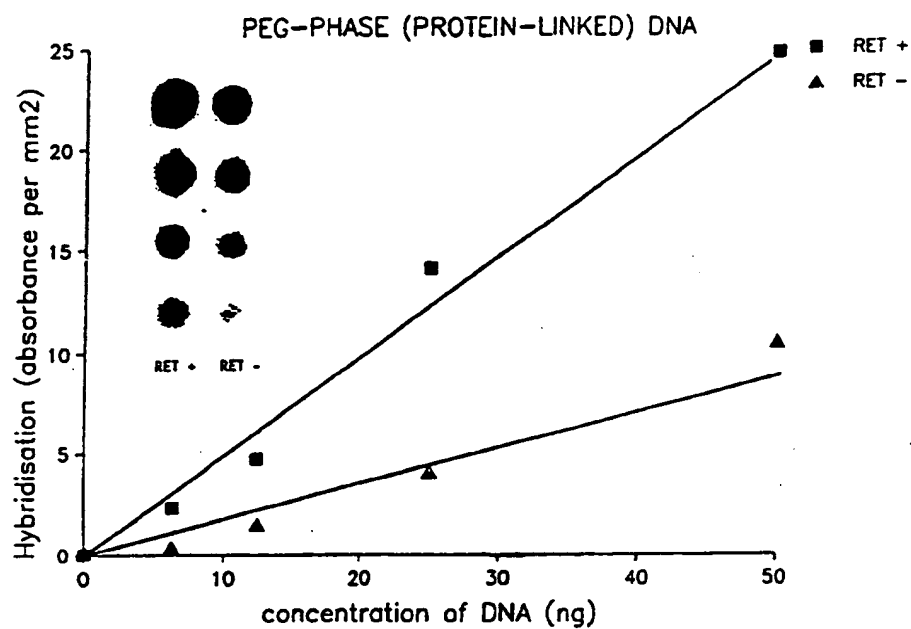


8/18

Fig. 10.*Fig. 11.*

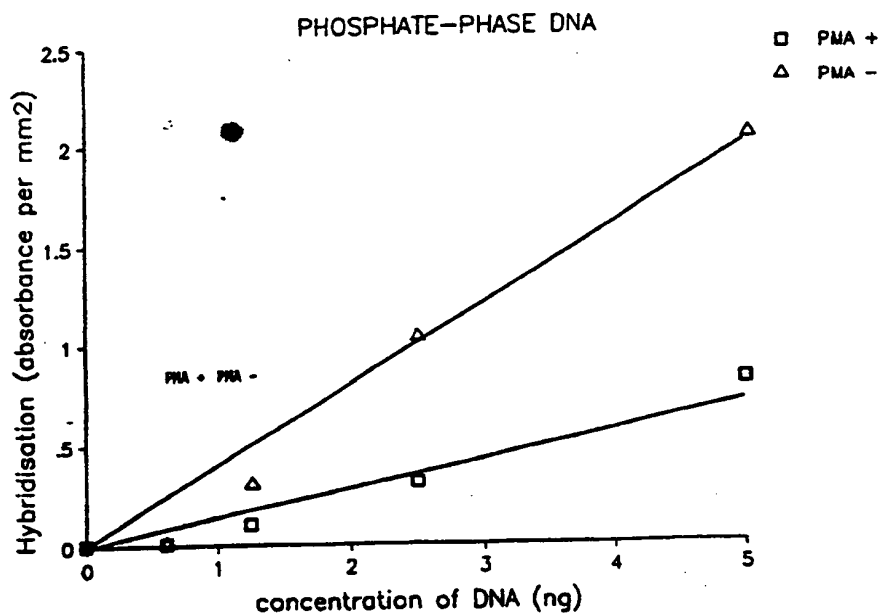
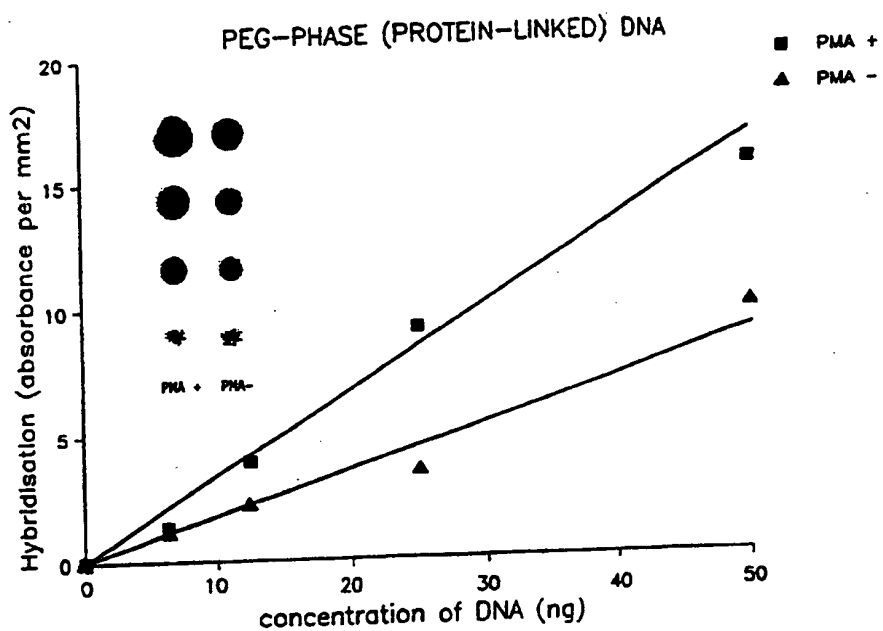
9/18

Fig.12.



10/18

Fig.13.



11/18

Fig. 15.

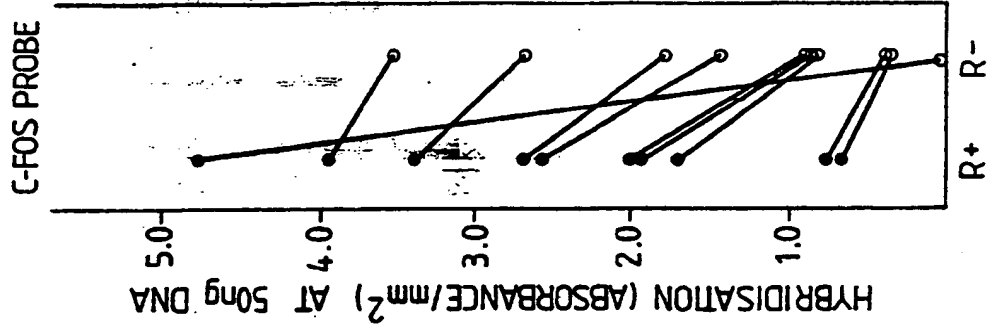
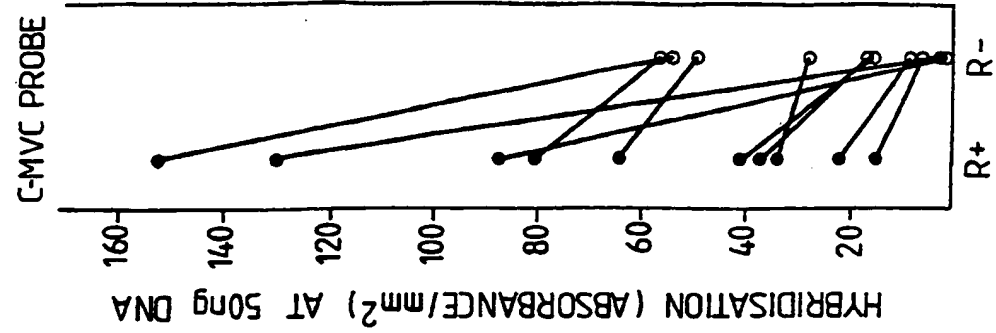
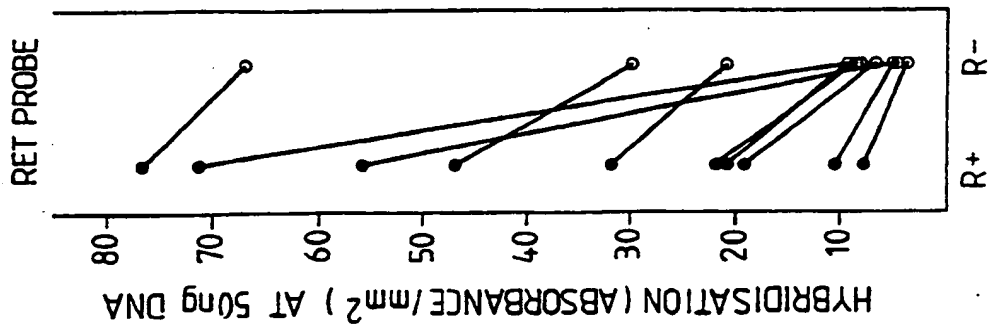
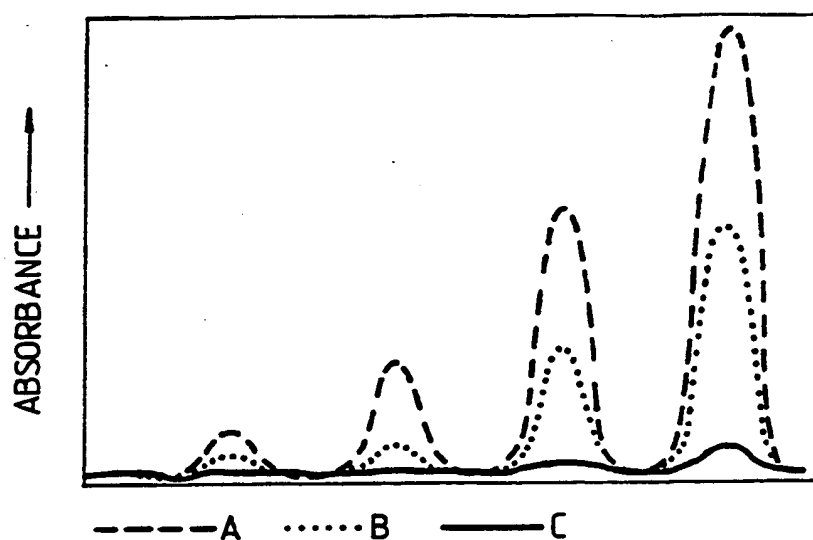
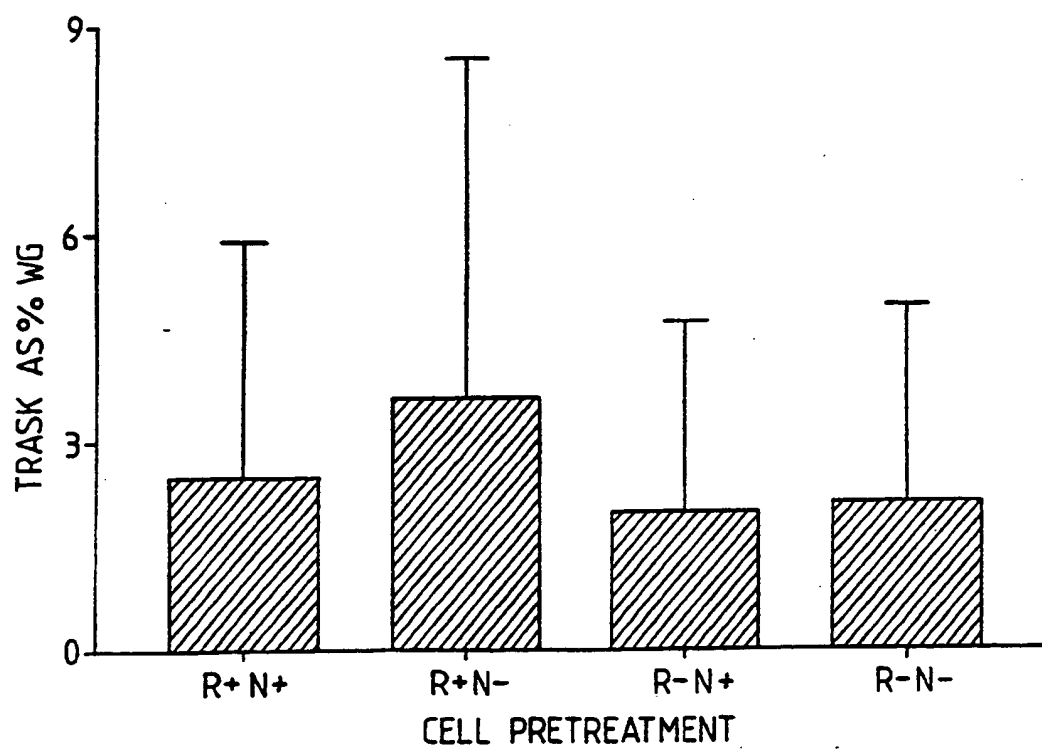


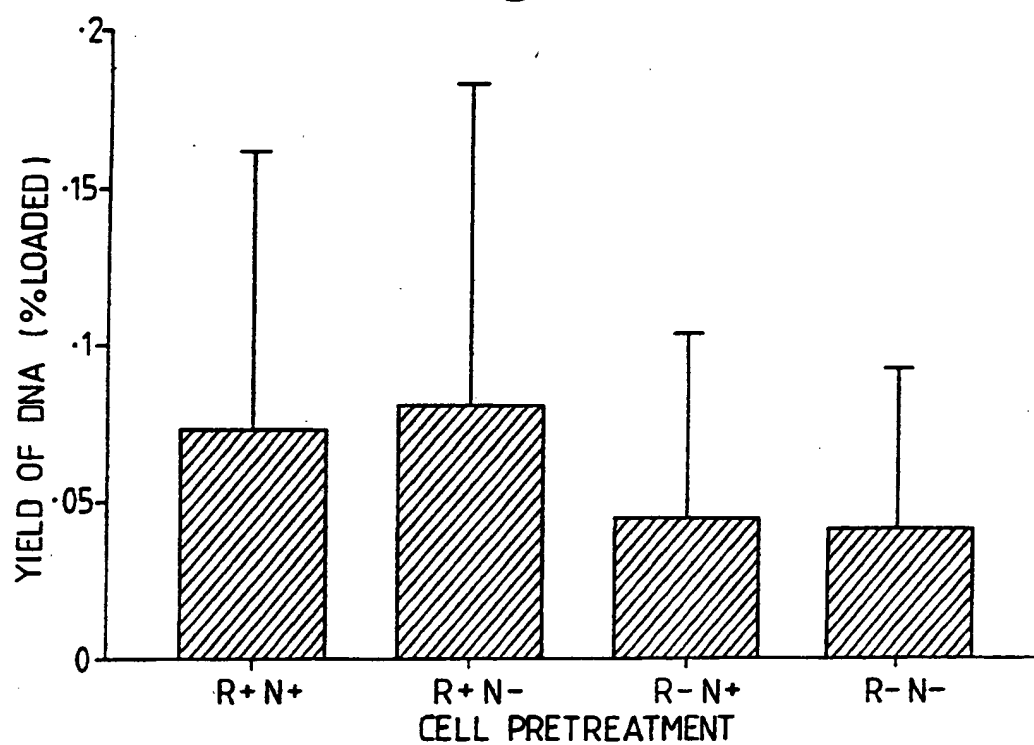
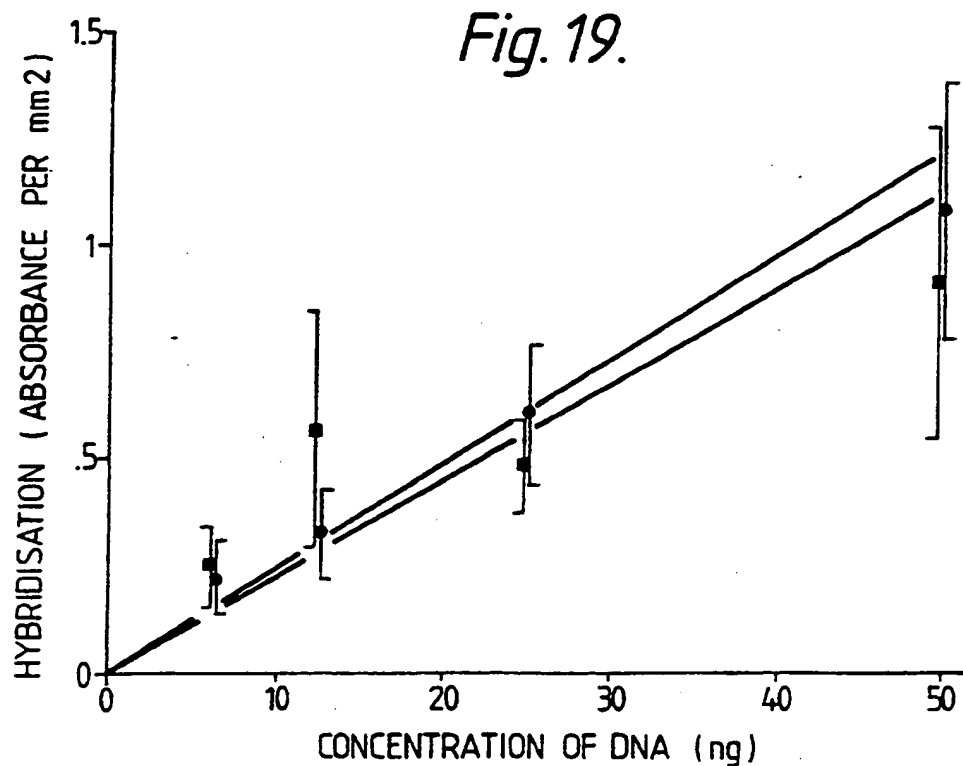
Fig. 14.



12/18

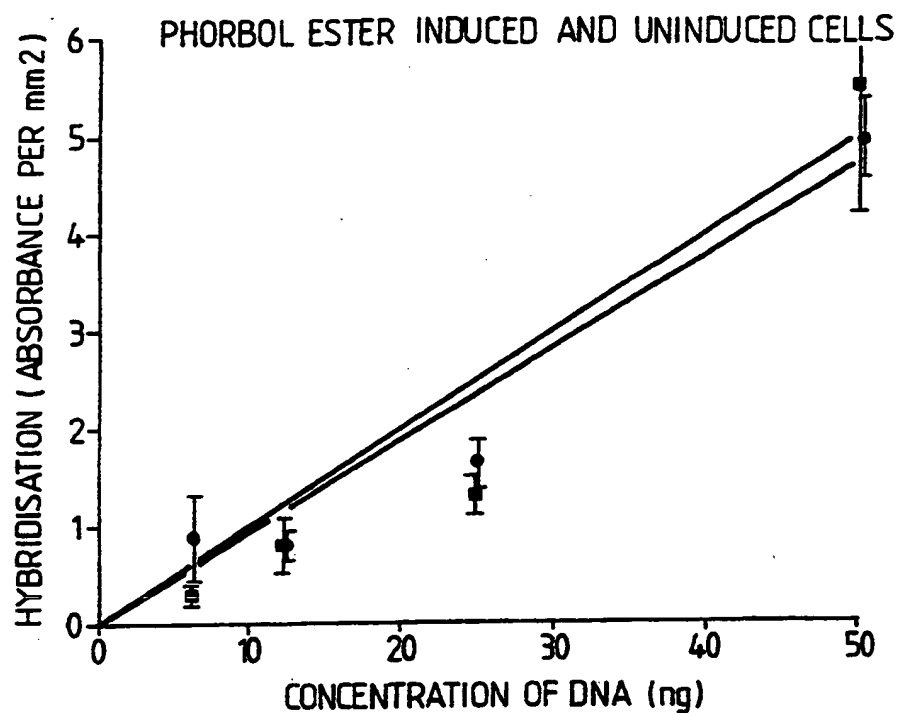
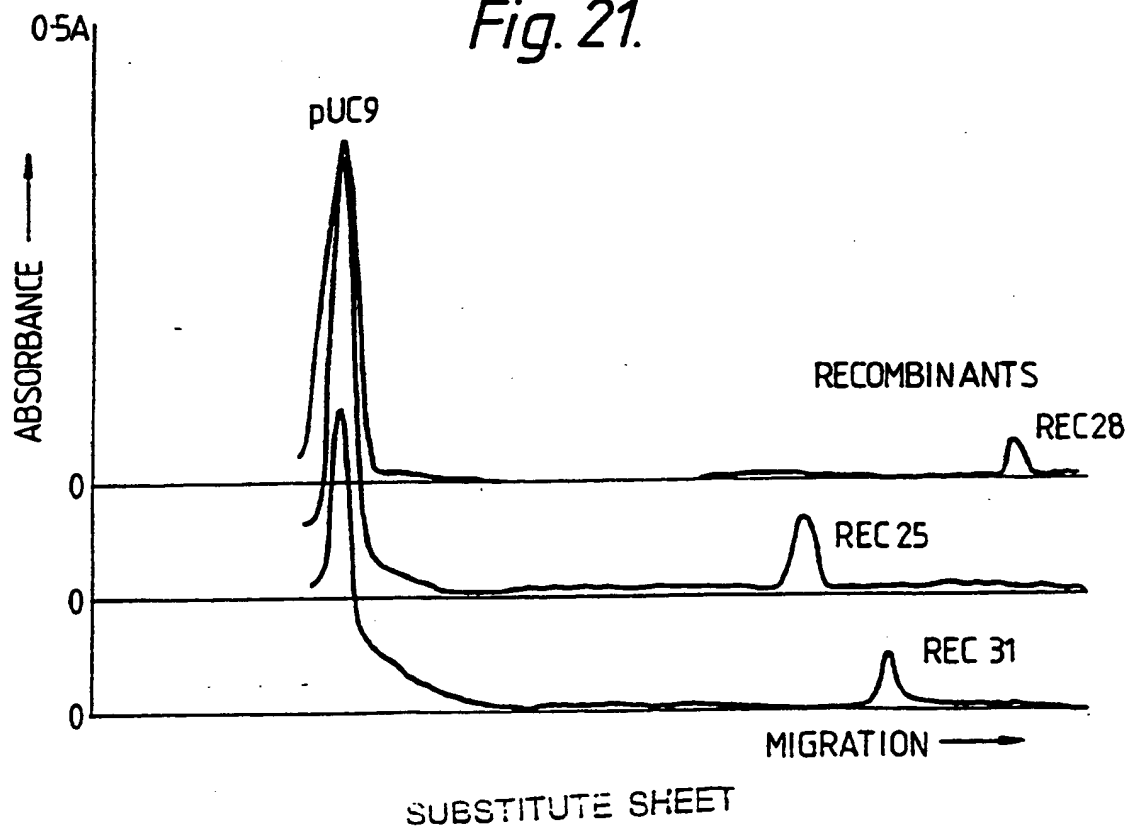
Fig. 16.*Fig. 17.*

13/18

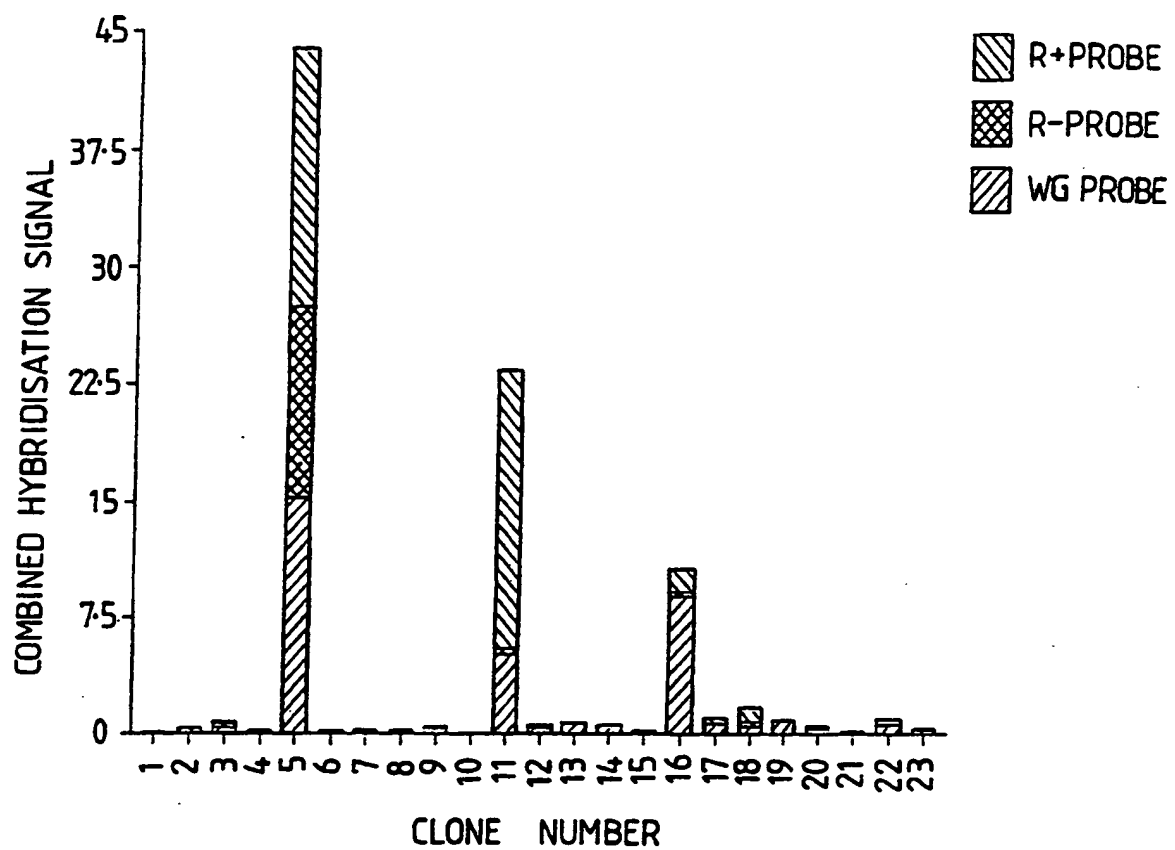
Fig. 18.*Fig. 19.*

SUBSTITUTE SHEET

14/18

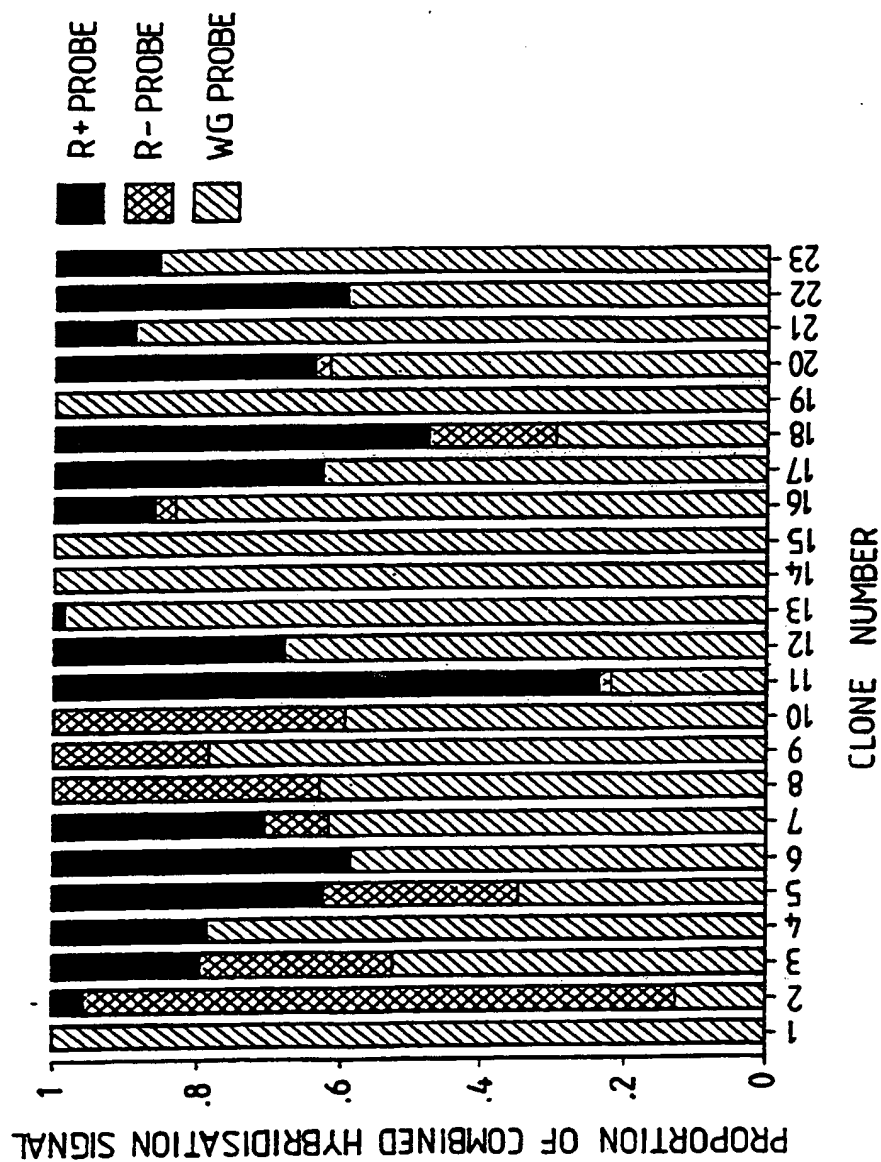
Fig. 20.*Fig. 21.*

15/18

Fig. 22.

16/18

Fig. 23.



17/18

Fig. 24.

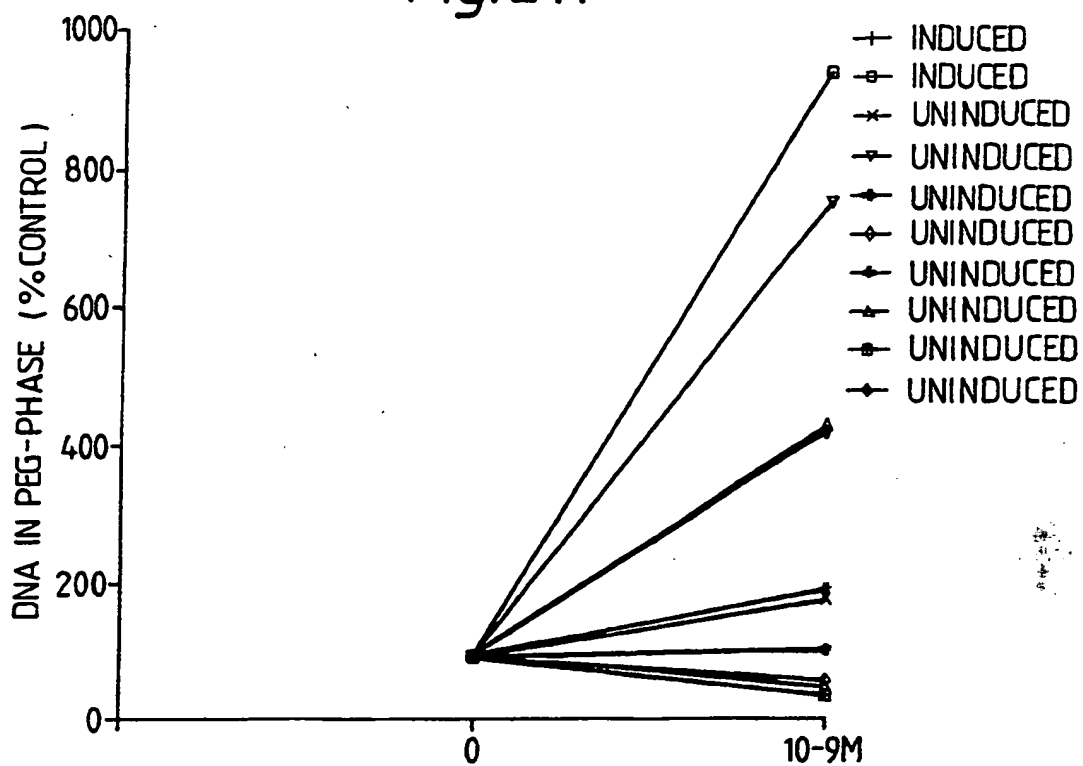
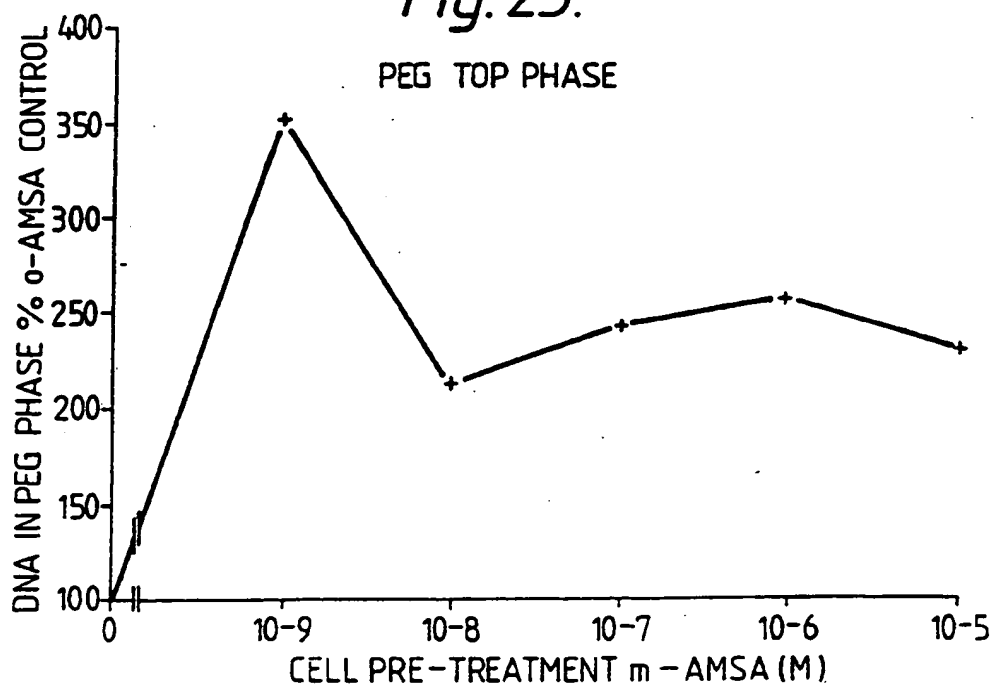


Fig. 25.



18/18

Fig. 26.

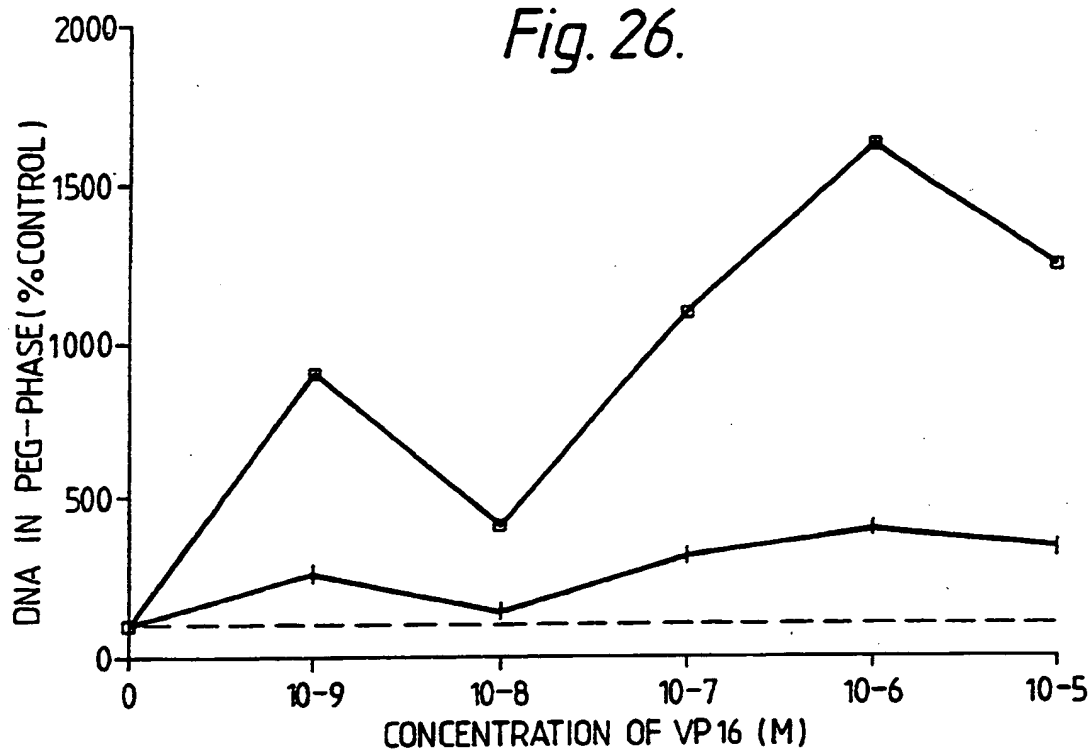
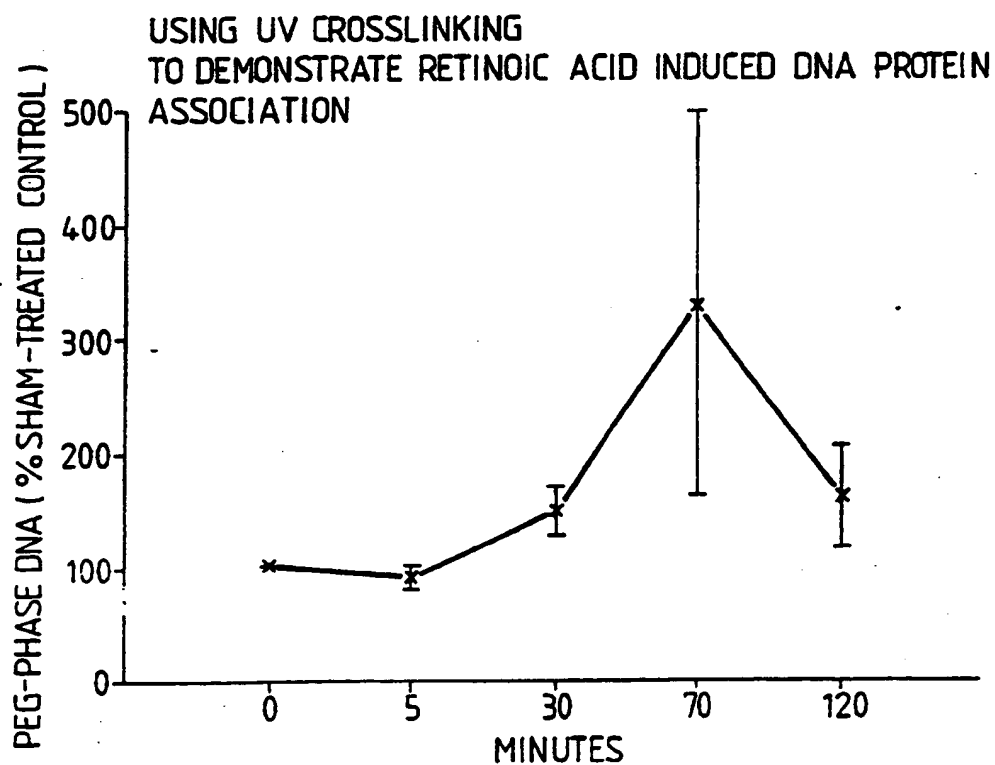


Fig. 27.



INTERNATIONAL SEARCH REPORT

International Application No. **PCT/GB 89/01263**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 Q 1/68														
II. FIELDS SEARCHED <div style="text-align: right; font-size: small;">Minimum Documentation Searched †</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">Classification System </td> <td style="width: 50%; border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; padding-top: 10px;">IPC5</td> <td style="border: none; padding-top: 10px;">C 12 Q</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ‡</div>			Classification System	Classification Symbols	IPC5	C 12 Q								
Classification System	Classification Symbols													
IPC5	C 12 Q													
III. DOCUMENTS CONSIDERED TO BE RELEVANT † <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th style="width: 10%;">Category *</th> <th style="width: 70%;">Citation of Document, †† with indication, where appropriate, of the relevant passages ‡‡</th> <th style="width: 20%;">Relevant to Claim No. ‡‡</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">The EMBO Journal, Vol. 3, No. 3, 1984, Douglas K. Trask et al.: "Rapid detection and isolation of covalent DNA/protein complexes: application to topoisomerase I and II.", see page 671 - page 676 --</td> <td style="text-align: center; vertical-align: top;">1-12</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">Biochemistry, Vol. 20, 1981, Leonard A. Zwelling et al.: "Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4'-(9-acridinylamino)methanesulfon-m-anisidide and adriamycin", see page 6553 - page 6563 --</td> <td style="text-align: center; vertical-align: top;">1-12</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">Chem. Pharm. Bull., Vol. 36, No. 8, 1988, Paul McGoff et al.: "Analysis of Polyethylene Glycol Modified Superoxide Dismutase by Chromatographic, Electrophoretic, Light Scattering, Chemical and Enzymatic Methods ", see page 3079 - page 3091</td> <td style="text-align: center; vertical-align: top;">1</td> </tr> </tbody> </table>			Category *	Citation of Document, †† with indication, where appropriate, of the relevant passages ‡‡	Relevant to Claim No. ‡‡	A	The EMBO Journal, Vol. 3, No. 3, 1984, Douglas K. Trask et al.: "Rapid detection and isolation of covalent DNA/protein complexes: application to topoisomerase I and II.", see page 671 - page 676 --	1-12	A	Biochemistry, Vol. 20, 1981, Leonard A. Zwelling et al.: "Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4'-(9-acridinylamino)methanesulfon-m-anisidide and adriamycin", see page 6553 - page 6563 --	1-12	A	Chem. Pharm. Bull., Vol. 36, No. 8, 1988, Paul McGoff et al.: "Analysis of Polyethylene Glycol Modified Superoxide Dismutase by Chromatographic, Electrophoretic, Light Scattering, Chemical and Enzymatic Methods ", see page 3079 - page 3091	1
Category *	Citation of Document, †† with indication, where appropriate, of the relevant passages ‡‡	Relevant to Claim No. ‡‡												
A	The EMBO Journal, Vol. 3, No. 3, 1984, Douglas K. Trask et al.: "Rapid detection and isolation of covalent DNA/protein complexes: application to topoisomerase I and II.", see page 671 - page 676 --	1-12												
A	Biochemistry, Vol. 20, 1981, Leonard A. Zwelling et al.: "Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4'-(9-acridinylamino)methanesulfon-m-anisidide and adriamycin", see page 6553 - page 6563 --	1-12												
A	Chem. Pharm. Bull., Vol. 36, No. 8, 1988, Paul McGoff et al.: "Analysis of Polyethylene Glycol Modified Superoxide Dismutase by Chromatographic, Electrophoretic, Light Scattering, Chemical and Enzymatic Methods ", see page 3079 - page 3091	1												
<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ††</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> Date of the Actual Completion of the International Search 10th January 1990 </td> <td style="width: 50%; border: none; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: large;">31 JAN 1990</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="border: none; vertical-align: top;"> Signature of Authorized Officer <div style="text-align: right; font-weight: bold; margin-top: 10px;">T.K. WILLIS</div> </td> </tr> </table>			Date of the Actual Completion of the International Search 10th January 1990	Date of Mailing of this International Search Report <div style="text-align: center; font-size: large;">31 JAN 1990</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: right; font-weight: bold; margin-top: 10px;">T.K. WILLIS</div>								
Date of the Actual Completion of the International Search 10th January 1990	Date of Mailing of this International Search Report <div style="text-align: center; font-size: large;">31 JAN 1990</div>													
International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: right; font-weight: bold; margin-top: 10px;">T.K. WILLIS</div>													

THIS PAGE BLANK (USPTO)